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THE GROWTH AND RESPIRATION OF THE AVENA COLEOPTILE

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I

INTRODUCTION

In a previous paper (1) a relation was shown to exist between the respiration of the plant cell and its elongation under the influence of the plant growth hormone. A more extensive investigation of this relation was therefore undertaken with the hope that elongation would exhibit a close correlation with some relatively accessible property of the respiration, for example with the magnitude or the respiratory quotient of the latter. It may be said at once, however, that this was not the case, and that the work reported in the present paper, while revealing several points of interest and defining more clearly the dependence of elongation upon respiration, has not resulted in any explanation of the way in which respiration is essential to growth.

II

Materials and Methods

Avena coleoptiles from seedlings of the pure line *Siegs Hafer* [1] were used throughout. They were grown under the usual controlled conditions (2). The measurements of respiration were made by the method of Warburg with the apparatus of Dr. Robert Emerson to whom the author is greatly indebted. Conical vessels possessing a central alkali well and side wells for reagents to be added during the course of an experiment were used. Twenty coleoptile sections (1) suspended in the desired solution were placed in each vessel. The measurements of elongation were carried out in the manner described in the previous paper (1).

1

III

Steps of the Growth Process Dependent upon Respiration

It was earlier shown that coleoptile sections do not elongate in growth substance solution when placed in an atmosphere of oxygen-free nitrogen   When such sections were subsequently placed in growth substance solution in air they elongated rapidly, showing that they had not been harmed   If they were, however, placed in water without growth substance they did not elongate, showing that they had not taken up and stored growth substance, that is, that the *transport* of the hormone into the sections does not take place in the absence of aerobic metabolism   In order to show that the action of growth

TABLE I

Inhibition of the Action of Growth Substance Already in the Plant

Agar block (2 hrs)	Subsequent solution	Subsequent elongation
		per cent in 8 hrs
Water agar	Distilled water	3 4
Water agar	10 u /cc	27 6
1200 plant units	Distilled water	12 8
1200 plant units	KCN, 3×10^{-3}N	2 1
1200 plant units	KCN, 1×10^{-3}N	3 7
1200 plant units	Phenylurethane, 0 05 per cent	4 9

substance already *in* in the section is also inhibited under these conditions, the following type of experiment was performed   Two lots of coleoptile sections were prepared   Upon the apical ends of one lot were placed agar blocks containing no growth substance   Upon the apical ends of the other set were placed agar blocks containing 1200 plant units of the hormone   The sections were left at 25°C in a saturated atmosphere for 2 hours   At the end of this time some of those which had had no growth substance were placed in water, the remainder in a solution containing 10 units of growth substance per cubic centimeter   The sections which had had 1200 plant unit blocks were distributed among the following solutions, KCN, 3×10^{-3}N, KCN, 1×10^{-3} N, phenylurethane, 0 05 per cent, water   Table I gives the results of a typical experiment   There is no question but that growth

substance passed into the sections which had had 1200 plant unit blocks, since these sections elongated more in water than did those which had had plain agar and were also placed in water This elongation brought about by growth substance already in the plant is, however, clearly inhibited by both KCN and phenylurethane We must conclude, therefore, that both growth substance *transport* and growth substance *action* are dependent upon aerobic metabolism

IV

Effect of Growth Substance upon the Magnitude of Respiration

In the earlier paper (1) it was shown that crude growth substance preparations cause an increase in the rate of respiration of coleoptile

TABLE II

Effect of Crystalline Auxine B on Coleoptile Respiration*

Solution	Q_{O_2} before addition	Q_{O_2} after addition
0 0 units *per cc*	0 60	0 60
10 units *per cc*	0 70	0 60

* In this and in all subsequent tables, Q_{O_2} denotes mm³ of oxygen consumed per section per hour, measured at standard conditions of temperature and pressure

sections It was suggested that although this increase might prove to be due to the hormone itself, it might on the other hand be due to associated impurities The latter supposition can now be shown to be the correct one since, as has also been found by Kogl (3) the respiratory stimulant is absent from crystalline auxine and can be removed from crude hetero auxine (4) preparations by further purification Table II shows that crystalline auxine B (kindly supplied by Professor F Kogl, Utrecht) does not affect coleoptile respiration Table III shows that while a crude hetero auxine preparation (from cultures of *Rhizopus suinus* (4)) having an activity of 500,000 plant units per milligram causes a considerable increase in respiration, a purification of this extract to 1,500,000 plant units per milligram removes most of the stimulating power

The respiratory effect of the more impure hormone preparation is

probably due to a specific stimulating substance rather than to a nutritive action of the associated impurities for the following reasons, (a) it has been found that fructose, which should also have a nutritive action, has but little effect upon coleoptile respiration under the conditions used and (b) it has been shown (1) that the stimulating substance is destroyed by oxidation with H_2O_2

That growth substance does not influence the magnitude of the respiration may be demonstrated in another manner   As has been described elsewhere (2) the activity of growth substance in promoting cell elongation depends upon the undissociated growth substance present   However, the pH of the cell contents is such that only 4 to 5 per cent of the hormone present in the plant is in the active form   If the cell acidity is increased by placing the coleoptile section in an acid

TABLE III

Effect of Crude Hetero-Auxine Preparations upon Respiration

Solution of	Q_{O_2} before addition	Q_{O_2} after addition	
		1 hour	2 hours
10 units *per cc* (2500 units, or 500,000 plant units, *per mg*)	1 56	1 92	1 95
10 units *per cc* (7500 units, or 1,500,000 plant units, *per mg*)	1 53	1 63	1 43

buffer solution, more of the cell's own growth substance becomes active and its rate of elongation is increased even without the addition of more growth substance   If, on the other hand, the section is placed in a neutral or basic buffer solution, the acidity of the cell contents becomes less than normal, growth substance is dissociated, and the rate of elongation of the section is decreased   A comparison of the respiratory rates in buffer solutions of pH 4 1 and pH 7 2 was therefore made   Since the buffer of pH 7 retains a portion of the CO_2 given off by the respiring tissue it was necessary to add an excess of acid at the end of each experiment, in order to drive off all of the accumulated CO_2 into the gas space of the vessel, where it could be absorbed by the KOH of the alkali well   Table IV shows that there is no difference in the respiratory rates at the two pH values although the rates of

elongation are greatly different It is clear, then, that growth sub stance has no effect upon the magnitude of coleoptile respiration, a conclusion also reached by Kogl (3)

Since changes in the growth rate of the coleoptile induced by growth substance are not paralleled by changes in the intensity of respiration

TABLE IV

Effect of pH upon the Elongation and the Respiration of Coleoptile Sections

	pH 4 1	pH 7.2
Elongation in 2 hrs *per cent*	9 4	2 7
Qo₂	1 53 ± 0 05	1 49 ± 0 05
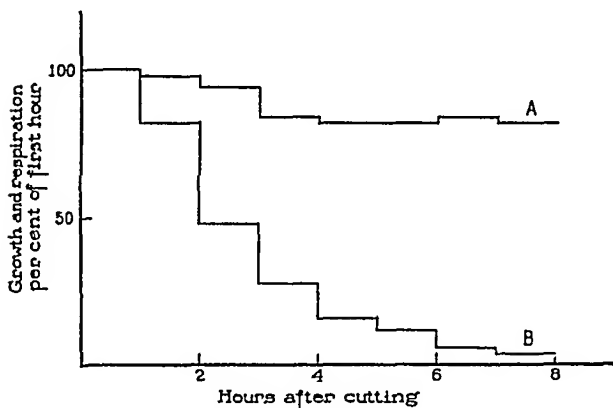
FIG 1 Changes in the growth rate and in the respiration rate of coleoptile sections with time Curve A, respiration rate, Curve B, growth rate

it might be expected that changes in the growth rate induced by other causes should also be without effect Fig 1 compares graphically the changes with time of (*a*) the growth rate, and (*b*) the rate of respira tion, of sections immersed in growth substance solution The growth rate even in the presence of excess growth substance decreases steadily

until after 8 hours it practically reaches 0   At this time then, substances or conditions other than the growth hormone are limiting elongation   The rate of respiration, on the other hand, sinks only about 20 per cent in this time   Similarly, the distribution of growth rate and respiratory rate over the length of the coleoptile do not parallel one another markedly, as is shown in Fig 2   Even when the
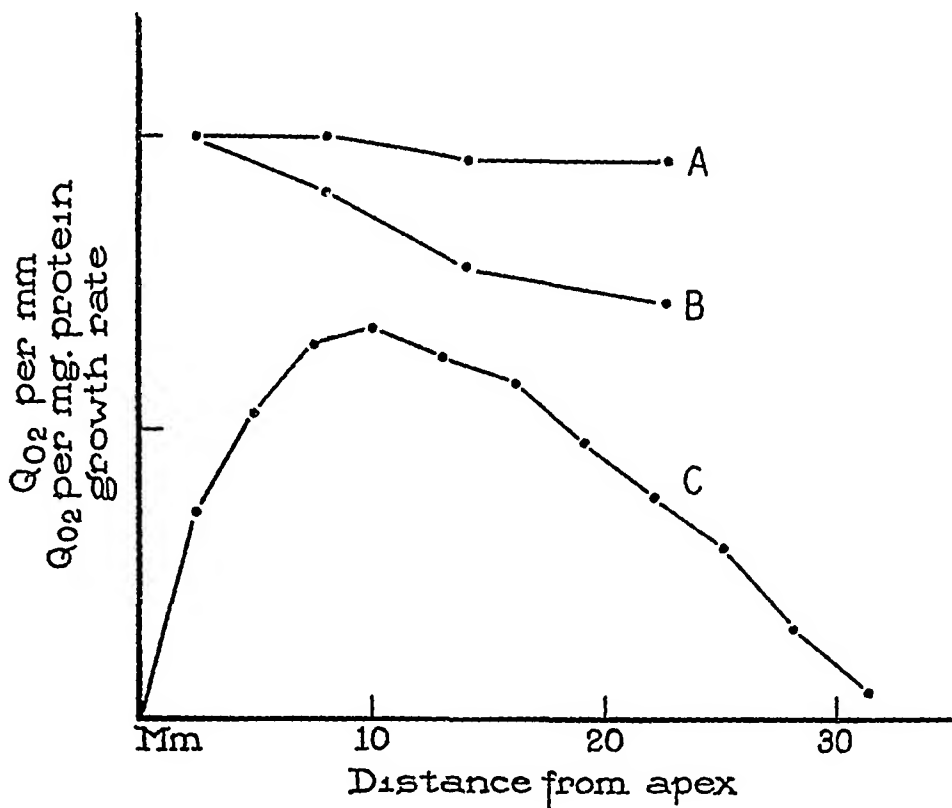
FIG 2 Distribution of growth rate and of respiration rate in the coleoptile Curve A, rate of respiration per milligram, Curve B, rate of respiration per milligram protein, Curve C, growth rate (after Went (9))

respiration per milligram protein is used as the basis of comparison, the respiration of the slowly growing base of the coleoptile is 80 per cent of that in the zone of maximum growth rate   Although there is in these two cases no strict parallelism between elongation and respiration, still it is to be noted that a slight decrease in respiratory rate is accompanied by a large decrease in growth rate

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Does Growth Substance Change the Nature of the Respiration?

A search for qualitative differences in the nature of the respiration in the presence and in the absence of growth substance was next made. The respiratory quotient of sections under different conditions was determined by the method of Warburg. This method consists in the measurement of the gas which may be driven out of the tissue by acid at the beginning of the experiment ("preformed CO_2"), the net gas exchange, the gas driven off by acid at the end of the experiment, and the oxygen consumption during the experiment. The determination

TABLE V

Effect of Growth Substance upon the Respiratory Quotient

Solution	Total gas exchange mm.³	O₂ uptake mm.³	R Q
pH 4 1	+51 3	175 0	1 29
pH 4 1 + growth substance	+51 2	175 0	1 29
pH 7 2	−41 1	243 0	0 83
pH 7 2 + growth substance	−43 5	243 0	0 82
1 per cent fructose	−9 1	228 0	0 96
1 per cent fructose + growth substance	−8 1	228 0	0 96

of the respiratory quotients revealed a peculiar fact which has, however,—so far as can be seen at present,—no relation to the action of growth substance, namely, that the respiratory quotient is markedly higher for sections suspended in an acidic buffer than for sections suspended in a neutral buffer. In buffer of pH 4 1 the quotients are very constantly greater than one, in buffer of pH 7 2 they are more irregular but are markedly below one. It must be emphasized that the fact that during the main body of the experiment most of the CO_2 is driven off by buffer of pH 4 1 and a portion of it retained by buffer of pH 7 2 does not affect directly the determination of the respiratory quotient since in both cases all of the CO_2 is ultimately driven off with excess acid. Moreover, this difference in respiratory quotient between acid and neutral solutions has no direct relation to growth, since, as is shown in Table V, in unbuffered fructose solution the quotient is the same irrespective of the elongation, and is practically

one, as would be expected   The sample experiment of Table V shows
that in buffer of pH 4 1 the R Q is greater than one, in buffer of pH 7 2
less than one, and in unbuffered solution approximately one, and that
in all three of these cases growth substance is without effect   In spite
of the unexplained difference between acidic and neutral solutions it
seems safe to believe that growth substance itself has no influence upon
the respiratory quotient
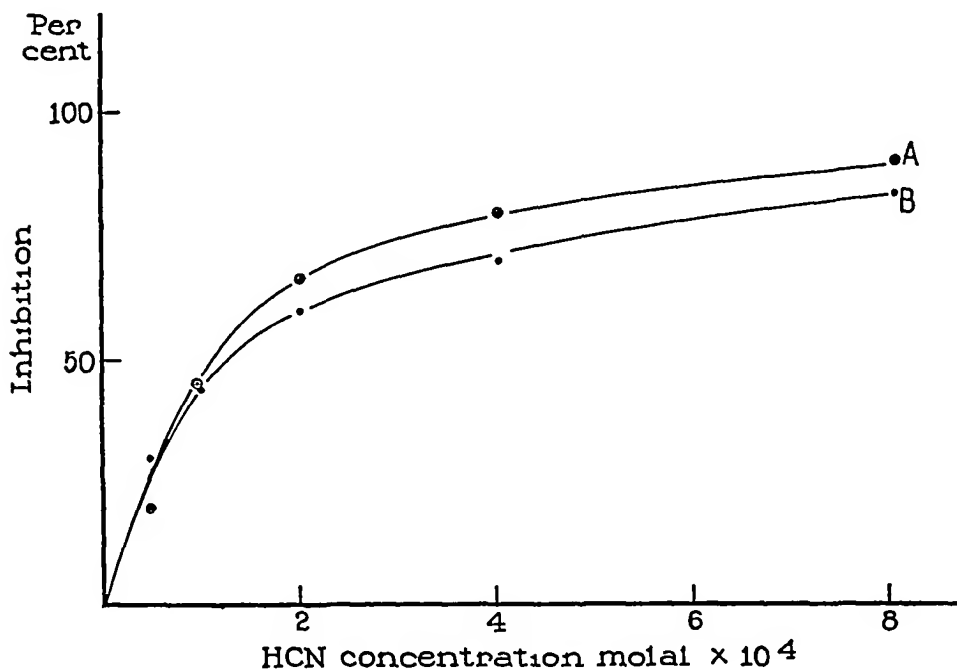
Fig 3 Inhibition of growth and of respiration by HCN   Curve A, inhibition
of respiration, Curve B, inhibition of growth

Strugger (5) has suggested that fermentation favors elongation
The high quotient at pH 4 might indeed be interpreted as a kind of
fermentation   However, this high quotient is not essential to growth
Moreover, if fermentation were conducive to cell elongation one might
expect that anaerobiosis should stimulate growth, or that HCN in
suitable amounts should, by inhibition of the "Pasteur reaction,"
increase elongation   An increase of growth rate by either anaerobic
conditions or HCN has never been observed in the course of these
experiments

VI

Effect of HCN on Growth and on Respiration

Up to the present point the independence, both quantitative and qualitative, of cell elongation and respiration has been stressed   In Part III and in the earlier paper (1) it has, however, been pointed out that this independence is by no means complete, since, for example, HCN concentrations of about the same order are needed to inhibit the two processes   A more quantitative study of this relation has now been made and the results are presented in Fig 3, in which per cent inhibition is plotted against HCN concentration   The two curves parallel one another very closely   A similar parallel inhibition of growth and of respiration was demonstrated earlier for phenylurethane (1)

VII

DISCUSSION

The experimental results which have been presented show that (a) during the growth of the cell, either in the normally growing coleoptile or in the excised coleoptile section, the rate of elongation decreases much more rapidly than does the rate of respiration, (b) HCN and phenylurethane decrease elongation and respiration to the same extent Low temperatures also slow down both processes, although respiration is somewhat more affected than is elongation (6)   Neutral buffers, however, decrease elongation without exerting any effect upon respiration

From (a) and (b) it is necessary to conclude that decreases in respiration decrease elongation but not the reverse, *i e*, no considerable portion of the respiration is the *result* of elongation processes   This dependence of growth upon a large respiration may be viewed in two ways, (1) the entire respiration is a "formal prerequisite" of growth (Pfeffer (7)), (2) one or more of the processes of growth is itself a respiratory process but one of small oxygen uptake   Calculations of the amount of mechanical work done during cell elongation have shown that only a small portion of the energy liberated during respiration can be used in this way, and many workers have shown that when the respiratory quotient is near one, the energy of respiration all

appears ultimately as heat (Algera (8))   (1) can give therefore no clear
idea of why a reduction of respiration by HCN or phenylurethane
should decrease elongation to even approximately the same extent
This brings up the question as to the way in which respiration might
be a formal prerequisite of growth   It is clear that it is necessary only
if it performs functions which are necessary for growth   The principal
function which it performs is the production of heat, which it is difficult
to visualize as useful for elongation   It seems more likely therefore
that only a portion of the respiration is needed in the actual growth
processes, and (1) would become identical with (2)

Experimentally also the distinction between (1) and (2) is difficult
if not impossible   (1) Growth is decreased when respiration is
decreased because the whole respiration is essential   (2) Growth is
decreased when respiration is decreased because the two portions of
the respiration have similar properties   Upon (1) elongation can
cease and respiration continue because the latter is a "prerequisite"
of the former   Upon (2) elongation can cease and respiration con-
tinue because of the inhibition of other portions of the growth process

Concept (1) therefore cannot be either theoretically or experi-
mentally strictly differentiated from concept (2)   It would seem
desirable then to avoid the ambiguity of meaning in statement (1),
and to express the necessity of respiration for growth in the statement
that a process of respiratory nature is one (or more) of the component
processes of elongation

VIII

SUMMARY

1 *Transport* of the plant growth hormone into the *Avena* coleoptile
as well as the *action* of the hormone on cell elongation in the coleoptile
are shown to depend upon aerobic metabolism

2 Crystalline auxine, in contrast with impure preparations, affects
neither the magnitude nor the respiratory quotient of coleoptile
respiration

3 Increasing age of the coleoptile cell decreases its rate of elongation
much more than its rate of respiration   HCN or phenylurethane on
the other hand decrease the two processes to the same extent, in spite

of the fact that only a small portion of the energy liberated by respiration can be used in the mechanical process of growth

4 From 2 and 3 it is concluded that processes of a respiratory nature but of relatively small magnitude form one or more integral steps in the chain of reactions by which the plant growth hormone brings about cell elongation

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CHANGES OF APPARENT IONIC MOBILITIES IN PROTOPLASM

I EFFECTS OF GUAIACOL ON VALONIA

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The order of apparent ionic mobilities[1] in *Valonia macrophysa*
Kütz is $K > Cl > Na$ This can be changed to $Na > Cl > K$
without injury to the cell, as is shown by experiments with guaiacol

Preliminary experiments were made by adding to sea water various
amounts of guaiacol (called HG for convenience) Exposures of a
few minutes at concentrations up to 0 03 M HG produced effects which
were completely reversible But 0 04 M HG was somewhat toxic and
as a rule no concentration above 0 02 M was employed Unless other
wise stated the experiments described in this paper produced no signs
of injury

METHODS

The procedure of Damon[2] was closely followed in all respects Cells were im
paled and left until the wound was healed The P D was measured by leading
off with calomel or Ag AgCl electrodes in the latter case one electrode was placed
in a narrow cup filled with sea water which was immersed in the solution surround
ing the cell thus keeping it in a constant concentration of Cl^- the other was
inserted in the capillary which pierced the cell The changes were recorded photo
graphically by means of a camera and a Cambridge Type A galvanometer[4] with
thermionic amplifier making the measurements essentially electrostatic in prin
ciple Calibrations were in all cases made by means of a potentiometer in series
with the cell

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[1] Damon E B *J Gen Physiol* 1932-33 **16**, 375 When I_{Cl} is taken as
unity $U_K = 20$ and $U_{Na} = 0 2$

For criteria of injury see Osterhout, W J V *Ergebn Physiol* 1933 **35**, 979
Kopac M J *Carnegie Institution of Washington Year Book No 32* 1932-33
273 *No 33* 1933-34 253

[2] Damon E B *J Gen Physiol* 1931-32 **15**, 525 (Fig 1)

[4] *Cf* Osterhout W J V and Hill S E *J Gen Physiol* 1933-34 **17**, 87

In making solutions of the pH of sea water indicator was added to the sea water which was then divided into two portions   To one of these there was added HG, followed by enough NaOH to bring the sea water back to the color it had at the start

With hydrogen and glass electrodes the liquid junction introduces an error of unknown magnitude [5]   It seemed best to neglect this   Accordingly, concentrated sea water was added to the standard buffer solutions[6] until their ionic strength was 0 721 which is the ionic strength of Bermuda sea water, according to Zscheile [7]   We thus avoid the salt error by making it approximately the same in the buffer and in the sea water   After adding the concentrated sea water the
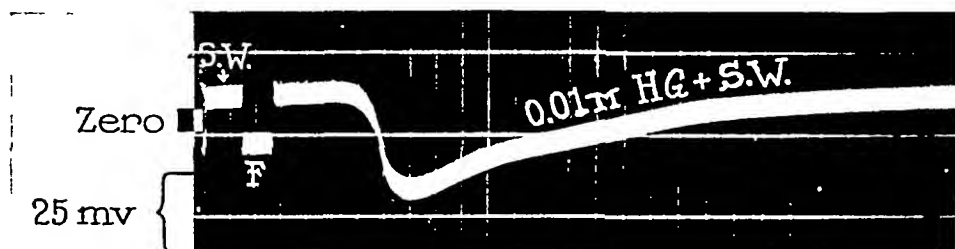
Fig 1 Photographic record showing the effect of 0 01 m HG in sea water at pH 6 4   The record starts with the cell in sea water   When it was lifted out the curve jumped down to the 'free grid" level, F, of the vacuum tube amplifier   The cell was then lowered into sea water plus 0 01 m HG at the instant it made contact with this solution the curve returned to its former level and there remained during the latent period (about 15 seconds), it then began to fall and became 26 mv more positive, after which it slowly rose and in about 100 seconds reached approximately its original level (for convenience the rise is termed "recovery" but this term does not signify that the cell has returned to its original condition)

The vertical marks are 5 seconds apart   Temperature 22°C

pH value of the buffer solution was determined by means of the hydrogen or glass electrode

Kahlbaum's guaiacol was used and the solutions were always freshly prepared No solutions were used in which color had developed   Unless otherwise noted all experiments were made with a collection of cells called Lot B   Lot C was not different in appearance but gave somewhat different results

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[5] It has been supposed that this could be rendered negligible by the use of saturated KCl but this has been called in question

[6] The buffers were phosphate and borate buffers (according to Clark, W M , The determination of hydrogen ions, Baltimore, The Williams & Wilkins Company, 3rd edition, 1928) and were approximately 0 2 m

[7] Zscheile, F P , Jr , *Protoplasma*, 1930, **11**, 481

Experiments with Sea Water

To avoid any complications due to the guaiacol ion (which may be called G^-) we begin with experiments at pH 6 4 where the concentration of G^- is negligible [6] This is desirable because in some cases the guaiacol ion appears to have considerable effect on the P D (by reason of its high mobility)

Fig 1 shows [9] what happens when a cell is transferred from sea water (at pH 6 4) to sea water [10] containing 0 01 M HG (at pH 6 4) At the start, the P D was 7 mv negative [11] (about the usual value) The HG caused it to become more positive by 26 mv (The mean of 33 measurements [1] was 28 mv, see Table I)

After this the curve returned to the previous level for convenience this will be referred to as "recovery" although it does not signify that the cell returned to its original condition The time required for the curve to fall and rise [13] (which will be called "recovery time") was variable the mean was 64 seconds, which was less than at pH 8 2 (there the value was 127 4 see Table I)

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[8] At pH 6 4 the degree of dissociation of HG in sea water is less than 0 1 per cent According to Shedlovsky and Uhlig (Shedlovsky, T and Uhlig H H, *J Gen Physiol* 1933-34 **17**, 567) the pK at 25 is 10 1 Subtracting 0 375 from this on account of the ionic strength of sea water (cf Jacques A G *J Gen Physiol* 1935-36 **19**, 397) gives 9 725

[9] Fig 1 is fairly typical but the form of the curve showed great variation In some cases the rise at the right end (comprising the last 10 to 15 mv) was quite steep In some cases (at pH 6 4 to 9 6) the curve dropped as usual then rose a little and fell before commencing the long steady rise resulting in recovery

[10] The cell had previously been transferred from sea water at pH 8 2 to sea water at pH 6 4 which had practically no effect on the P D

[11] *I e* the positive current tended to flow from the sea water across the protoplasm into the sap In some cases the P D was positive at the start but this seemed to make no difference in the behavior of the cells Regarding the P D of the cells in sea water see Blinks L R *J Gen Physiol* 1935-36 **19**, 633

[1] A few exceptional cells (not included in these measurements) gave much higher values (up to 106 mv) Lots D and E gave average values of about 65 mv Some cells in each lot gave no response but such cells sometimes responded on the following day

[13] This time is measured from the instant when the curve starts to fall to the time when it has risen again to its final level which is usually not much below the level at the start and may coincide with it as in Fig 1

TABLE I

Effects of Guaiacol*

Lot B except as otherwise stated   (The number of observations is given in parentheses)

pH	Solutions (sea water = S W)	Latent period	Change in P D	Recovery† time
		sec	mv	sec
6 4	S W then S W + 0 01 M HG	17 4 ±0 5 (35)	+28 0 ±2 1 (33)	64 0 ±2 3 (26)
8 2	S W then S W + 0 005 M HG	23 4 ±0 5 (13)	+22 3 ±2 8 (15)	—
	S W then S W + 0 01 M HG	15 6 ±0 6 (30)	+26 2 ±1 7 (30)	127 4 ±9 5 (14)
	S W + 0 005 M HG then S W + 0 01 M HG	0	+17 9 ±2 6 (12)	—
	S W + 0 01 M HG then S W + 0 02 M HG	0	+17 2 ±1 8 (15)	—
	S W + 0 02 M HG then S W + 0 04 M HG	0	+8 8 ±0 8 (5)	—
	S W then S W + 0 005 M KG	21 3 ±0 3 (10)	+14 6 ±0 1 (5)	—
	S W + 0 005 M KG then S W + 0 01 M KG	0	+21 9 ±3 3 (7)	—
	S W + 0 01 M KG then S W + 0 02 M KG	0	+26 6 ±2 0 (11)	—
	S W + 0 005 M HG then S W + 0 02 M HG	0	+34 5 ±3 4 (11)	—
6 4	0 6 M NaCl then 0 6 M NaCl + 0 01 M HG	22 0 ±0 9 (25)	+53 5 ±3 5 (25)	76 0 ±6 8 (5)

<div align="center">Lot C</div>

pH	Solutions (sea water = S W)	Latent period	Change in P D	Recovery† time
6 4	S W then S W + 0 005 M HG	12 1 ±0 7 (7)	+28 0 ±2 7 (6)	125 0 ±8 6 (6)
	S W then S W + 0 01 M HG	10 3 ±0 5 (7)	+60 4 ±3 0 (6)	395 0 ±77 1 (6)
	S W then S W + 0 02 M HG	6 5 ±0 36 (4)	+83 4 ±3 4 (6)	955 0 ±65 6 (6)

*In making averages some extremely high and extremely low values were disregarded

† Includes time for fall of curve and rise to final level which may be the same as at the start or a little lower

In these experiments with 0 01 M HG it was observed that the greater the positive drop the longer is the recovery time When the concentration of HG is increased the positive drop increases and recovery is slower (Table I, I or C) and less complete Recovery may act thus because increase of concentration does not affect I as much as V in respect to speed or final effect Similar considerations may apply when one cell shows a greater positive drop and recovery time than another though both are exposed to the same concentration of HG

After applying HG there was a latent period[14] in Fig 1 of about 15 seconds before the curve began to fall The average of 35 measurements gave 17 4 (Table I) This is also seen at pH 8 2 with 0 005 M at pH 8 2 the latent period was 23 4 seconds and with 0 01 M (at pH 8 2) the mean was 15 6 seconds See also the measurements with I or C (Table I) Evidently the latent period shortens as the concentration increases

There is practically no latent period when sea water + 0 01 M HG is substituted for sea water + 0 005 M HG This is illustrated in Fig 2 When sea water was replaced by sea water containing 0 005 M guaiacol there was a positive movement of 15 mv Sea water containing 0 01 M guaiacol was then applied making the P D 27 5 mv more positive[15] Subsequent application of sea water containing 0 02 M guaiacol made the P D still more positive by 14 5 mv When replaced in sea water the P D returned to the value it had at the start There was no indication of latent period in applying 0 01 or 0 02 M HG

An interesting feature is the threshold Cells showing no effect with 0 005 M may respond when the concentration is raised to 0 01 M or higher (Fig 3)

There was no evidence of injury The cells when returned to sea water quickly regained their usual P D and many of them were used for several days in succession

[14] In some cases bacteria form a gelatinous covering over the cell which delays the entrance of electrolytes This was not the case with these cells as shown by the very prompt response when they were placed in sea water containing 0 5 M KCl or when the solutions were diluted (Figs 5 6, 9 11 and 12)

[15] Some exceptional cells gave very high values (up to 106 mv) when 0 01 M guaiacol was added directly to sea water
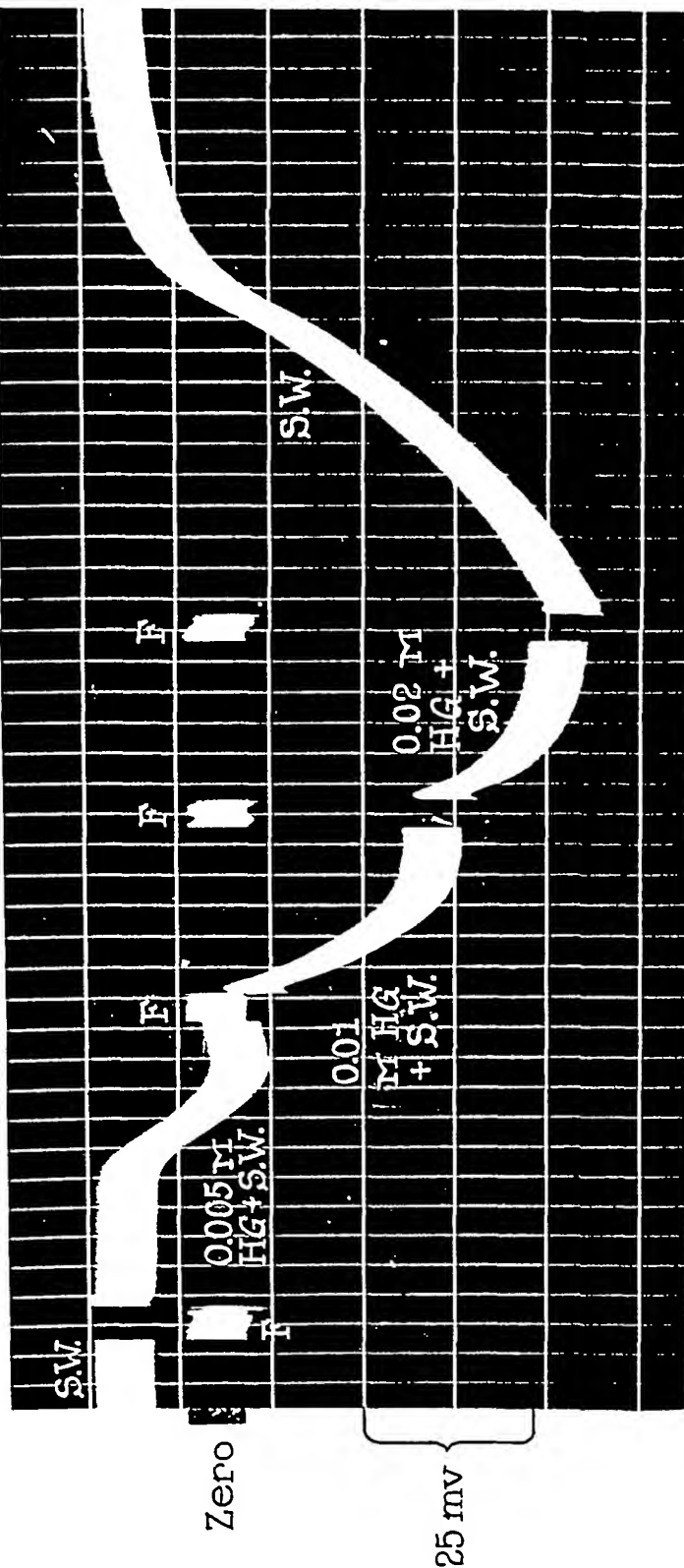
Fig 2 Photographic record showing the effects of increasing concentrations of HG (all at pH 8 2) The cell was at first in sea water and was then transferred to 0 005 M HG in sea water After a latent period of about 23 seconds the P D became 15 mv more positive and the curve then began to rise (recovery) Application of 0 01 M HG in sea water made the positivity greater by 27 5 mv than the greatest value attained in 0 005 M a further increase in positivity of 14 5 mv occurred in 0 02 M HG in sea water Replaced in sea water the P D returned approximately to its value at the start

Time marks are 5 seconds apart Temperature 23°C

Regarding F see Fig 1 (p 14)

18
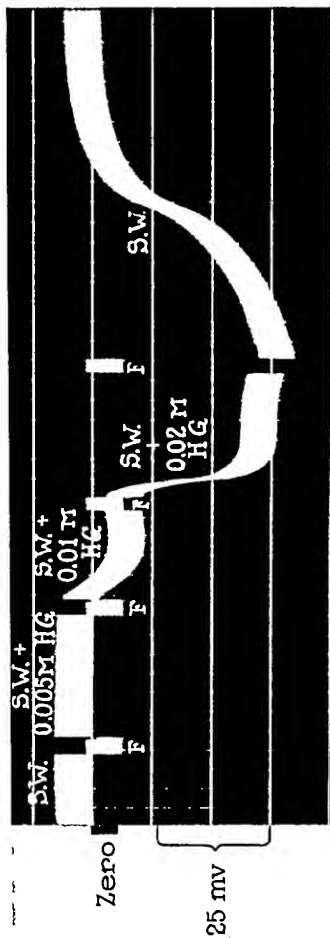
FIG 3 Photographic record showing that when 0 005 M HCl has been applied without effect, positivity can be produced by 0 01 M HG and there is no latent period (all solutions at pH 6 4)

At the start the cell was in sea water and when transferred to 0 005 M HG in sea water its p d remained practically unchanged It was then lifted out of the solution and the curve jumped down to the free grid level I of the vacuum tube amplifier The cell was then lowered into 0 01 M HG in sea water and the instant it touched this solution the curve jumped back to its former level (approximately) and at once begun to fall thus showing that there was no latent period (compare with Fig 1) On transferring to 0 02 M HG in sea water there was a further drop (unusually large see Table I) Replaced in sea water the p d returned approximately to its original value

In cells of this sort no positivity is produced by prolonging the exposure to 0 005 M HG
The vertical marks are 5 seconds apart Temperature 22°C

The most satisfactory way of dealing with these p d 's is to regard them as diffusion potentials [1] Hence when HG added to sea water or to NaCl produces positivity it is natural to inquire whether this depends on a decrease[16] in U_{Na} (the apparent mobility of Na$^+$ in the outer protoplasmic surface) as compared with V_{Cl} (the apparent mobility of Cl$^-$)

To answer this question experiments were made with 0 6 M NaCl which is nearly isotonic with Bermuda sea water

Experiments with NaCl[17]

The curve produced by exposure to 0 01 M HG in 0 6 M NaCl at pH 6 4 is much the same as that in Fig 1 Evidently the presence of the sea water ions, K$^+$, Mg^{++}, Ca^{++}, and SO$_4^-$, in the external solution is not necessary to produce this effect The concentration of these ions in the outer part of the protoplasm is doubtless lessened, especially when the cell is left for 2 or 3 minutes in 0 6 M NaCl before HG is applied

The next step was to measure the concentration effect in NaCl by transferring from 0 6 M NaCl[18] to 0 6 M NaCl plus 1 volume of 8 7 per cent glycerol (by weight) which is nearly isotonic with 0 6 M NaCl[1] As seen in Fig 4, in the dilute solution the P D becomes more

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[16] When V_{Cl} is greater than U_{Na}, so that Cl$^-$ tends to enter the protoplasmic surface more rapidly than Na$^+$, the solution tends to become more positive (in the external circuit) the greater its concentration Hence by diluting a solution of NaCl we can ascertain whether V_{Cl} is greater than U_{Na} for if it is the more dilute solution will be less positive (i e more negative) Cf Osterhout, W J V , *J Gen Physiol* , 1929–30, **13**, 715

[17] The experiments described in this section did not seem to produce any injury The cells remained normal in appearance and after replacement in sea water they quickly regained their normal P D Many of the impaled cells could be used for several days in succession

Changing from sea water to 0 6 M NaCl (with or without change of pH) has little or no effect [19] The amount of K$^+$ in the sea water is too small to have much effect on the P D (Damon, E B , unpublished results) and this applies to the other ions except Na$^+$ and Cl$^-$

[18] In the case of dead cells (i e impaled cells killed in position) dilution of 0 6 M NaCl or of sea water or of 0 6 M KCl, or of "sea water with 0 5 M KCl" with an equal volume of 8 7 per cent glycerol produced only a negligible change in P D

negative by 6 mv the average of 10 measurements was 8 4 mv
Since the more dilute solution is the more negative we conclude that
$U_{Na} < V_{Cl}$ (Table II)

After an exposure of 290 seconds to 0 01 M HG the concentration
effect was reversed so that the dilute solution became positive, in
dicating that $U_{Na} > V_{Cl}$ (Fig 5)
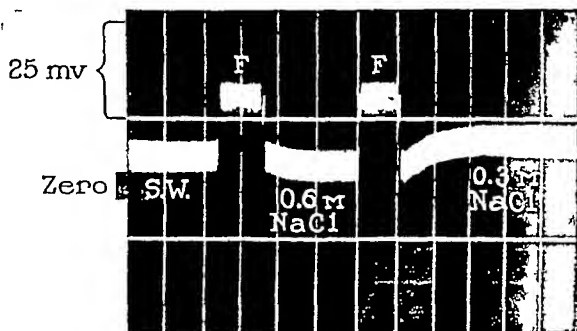
FIG 4 Photographic record showing normal concentration effect of NaCl
(all solutions at pH 6 4) The record starts with the cell in sea water the cell
was transferred to 0 6 M NaCl On diluting this with an equal volume of 8 7
per cent glycerol the P D became 6 mv more negative indicating that the apparent
mobility of Na+ was less than that of Cl− The cell was afterward returned to sea
water (not shown on the record) and behaved normally
    Time marks are 5 seconds apart   Temperature 24°C
    Regarding F see Fig 1 (p 14)

To minimize the danger of injury this longer exposure to HG was
carried out in sea water at pH 6 4 (a curve similar to that in Fig 1
was obtained during this process) The cell was then transferred to
0 6 M NaCl at pH 6 4 with the result shown in Fig 5 When this
NaCl was diluted with an equal volume of 8 7 per cent glycerol (con
taining 0 01 M HG) the P D became 15 mv more positive [19] In

[19] This reversal could be obtained equally well regardless of whether the cells
had previously been exposed to dilute solutions

FIG 5 Photographic record showing reversal of the concentration effect of NaCl after exposure to HG (all solutions at pH 6 4) The record begins with the cell in sea water + 0 01 M HG in which it had already remained for 290 seconds It was transferred to 0 01 M HG + 0 6 M NaCl Ihis solution was then diluted with an equal volume of 8 7 per cent glycerol + 0 01 M HG and the p D became 15 mv more positive (instead of negative as in Fig 4), indicating that the apparent mobility of Na+ had become greater than that of Cl− The cell was then returned to 0 01 M HG + 0 6 M NaCl and then to sea water

The vertical marks are 5 seconds apart   Temperature 24°C
Regarding F see Fig 1 (p 14)

some cases no change occurred [0] which may be taken as indicating that $U_{Na} = V_{Cl}$, aside from these cases we have 9 observations showing an average positive movement of 11 1 mv (Table II). To calculate

TABLE II

Concentration Effects (1) Normal and (2) Reversal Due to Exposure to Guaiacol I of B (The number of observations is given in parentheses)

pH	Solutions employed	Change of P D
	(1) Normal	*m*
8 2	S W then S W + equal volume glycerol 8 7 per cent	−10 2 ±0 4 (19)
6 4	S W then S W + equal volume glycerol 8 " per cent	−10 6 ±0 5 (7)
6 4	0 6 M NaCl then same + equal volume glycerol 8 7 per cent	−8 4 ±0 8 (10)
	(2) Reversal after 5 minutes or less in S W + 0 01 M HG	
6 4	S W + 0 01 M HG then same + equal volume glycerol 8 7 per cent containing 0 01 M HG	+13 0 ±1 7 (8)
6 4	0 6 M NaCl + 0 01 M HG then same + equal volume glycerol 8 7 per cent containing 0 01 M HG	+11 1 ±1 7 (9)
6 4	0 6 M KCl + 0 01 M HG then same + equal volume glycerol 8 7 per cent containing 0 01 M HG	−8 2 ±1 1 (6)
8 2	S W with 0 5 M KCl + 0 01 M HG then same + 2 volumes glycerol 8 7 per cent containing 0 01 M HG	−12 9 ±1 4 (6)
6 4	S W with 0 5 M KCl + 0 01 M HG then same + 2 volumes glycerol 8 7 per cent containing 0 01 M HG	−10 6 ±1 4 (8)
8 2	S W + 0 01 M HG then S W with 0 5 M KCl + 0 01 M HG	+20 4 ±1 8 (6)

the value of U_{Na} we may employ the formula [1]

$$P D = 58 \frac{V - U}{V + U} \log \frac{C_1}{C_2}$$

where $U = U_{Na}$, $V = V_{Cl}$, and C_1 and C_2 are concentrations (for our present purpose the activity coefficients may be left out of account)

[0] When no change occurred the question arose whether the cell was in such condition as to be incapable of showing any immediate change of P D as would happen if the outer surface were destroyed or short-circuited by leaks. This was ruled out in all cases by placing the cell in 0 6 M KCl which produced a prompt response

[21] Damon E B, and Osterhout W J V J Gen Physiol 1929–30 **13**, 445

Before treatment with HG the change due to 0 3 M NaCl is -8 4 from which (putting $V_{Cl} = 1$) we get $U_{Na} = 0$ 35   After treatment the P D is $+11$ 1 from which $U_{Na} = 4$ 5

The Concentration Effect with Sea Water[17]

Since the concentration effect of sea water is essentially that of NaCl[21] it is not surprising that it can be reversed like that of NaCl Cells were first tested for the normal effect as shown in Fig 6   Transferring from sea water to sea water plus 1 volume of 8 7 per cent glycerol gave as the average of 19 measurements at pH 8 2 a negative movement of 10 2 mv (Table II)   7 measurements at pH 6 4 gave 10 6 mv which is not far from Damon's[22] value[23] of 11 4 mv   As the dilute solution is more negative we may conclude that $U_{Na} < V_{Cl}$

After being tested in this way the cells were allowed to remain in sea water for several hours   Then they were placed for 5 minutes in sea water containing 0 01 M HG   The concentration effect was then found to be reversed (Fig 7), i e , the more dilute solution was positive[19] to the extent of 5 to 15 mv   In a few cases no change of P D occurred[20] which may be interpreted to mean that $U_{Na} = V_{Cl}$ omitting these we have 8 observations (Table II) giving for the positive movement an average value of 13 mv (the highest was 27 mv)   Before treatment with HG the P D of the dilute sea water was -10 6 from which we calculate $U_{Na} = 0$ 24   after treatment the P D was $+13$ 0, giving $U_{Na} = 7$ 0

Experiments with KCl[23]

When cells are transferred from sea water at pH 8 2 or 6 4 to "sea water with 0 5 M KCl" (1 part sea water plus 5 parts of 0 6 M KCl) there is a rapid increase in negativity[24] (Fig 8)   Replacing this solution with the same plus 1 part 8 7 per cent glycerol makes the P D less negative[25]   This is also true when we employ 0 6 M KCl in

[22] The response was more rapid than in Damon's experiments

[23] Prolonged exposure to 0 6 M KCl may produce injury but there was no sign of injury in these experiments

[24] The increase was more rapid than in Damon's experiments[1] but the amount of negativity was usually less

[25] This agrees with Damon's unpublished experiments
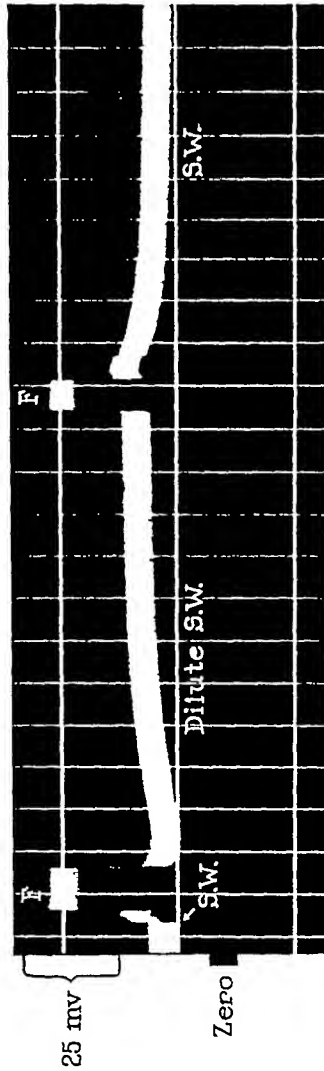
FIG 6 Photographic record showing the normal concentration effect of sea water (all solutions at pH 8 2) The record starts with the cell in sea water On diluting the sea water with an equal volume of 8 7 per cent glycerol the p.d became 6 mv more negative indicating that the apparent mobility of Na^+ was less than that of Cl^- The p.d returned to its initial value when the cell was replaced in sea water The vertical marks are 5 seconds apart Temperature 24°C

Regarding I see Fig 1 (p 14)
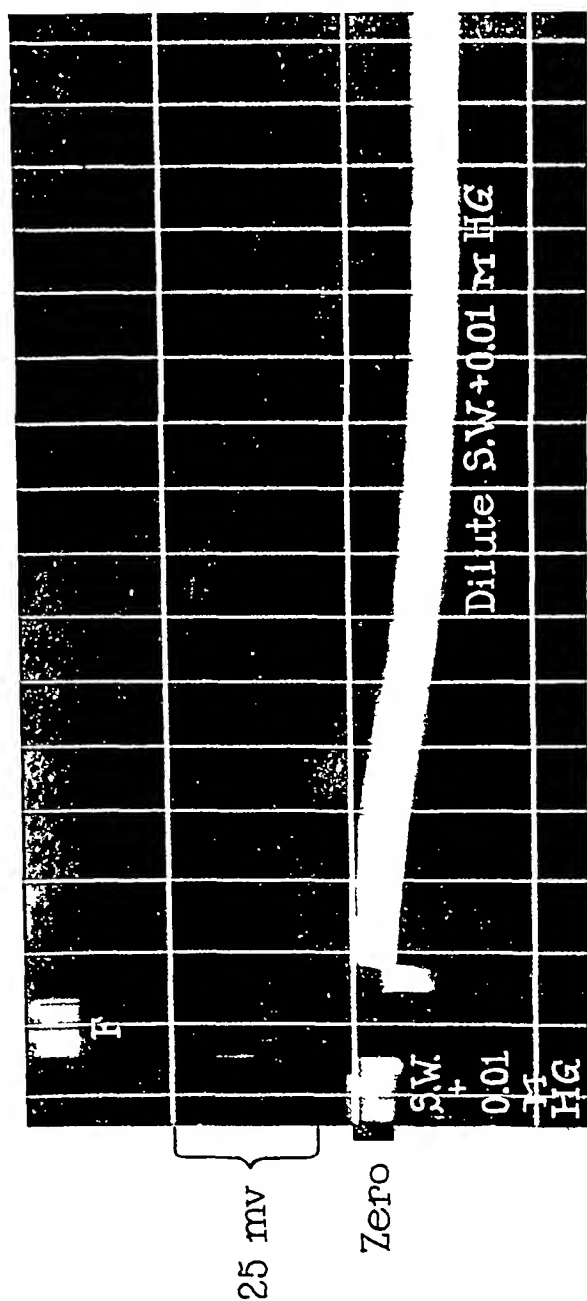
Fig 7 Photographic record showing reversal of the concentration effect of sea water after exposure to HG (all solutions at pH 8 2) The record starts with the cell in sea water containing 0 01 M HG in which it had been standing for 247 seconds On diluting this with an equal volume of 8 7 per cent glycerol + 0 01 M HG the P D became 16 mv more positive (instead of more negative, as in Fig 6), indicating that the apparent mobility of Na⁺ had become greater than that of Cl⁻ The P D returned to the normal value when the cell was replaced in sea water

The vertical marks are 5 seconds apart   Temperature 24°C
Regarding F see Fig 1 (p 14)

place of 'sea water with 0 5 M KCl'. Since the dilute solution is positive we conclude that $U_K > V_{Cl}$

In order to ascertain the effect of HG on U_K the concentration effect was studied after exposure to HG. Fig 9 shows an experiment with a cell which was transferred through a series of solutions (all at pH 6 4. On passing from sea water to 'sea water with 0 5 M KCl' the P D became 35 mv more negative. It returned to the original value when the cell
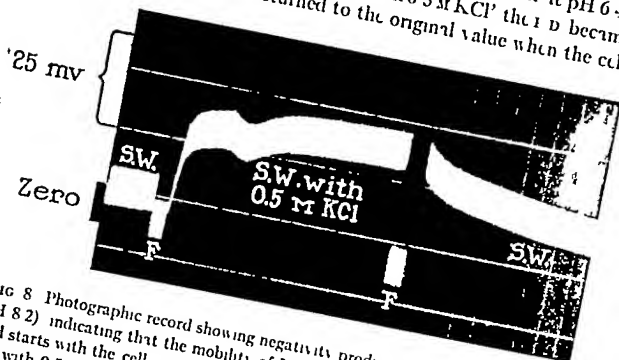
Fig 8 Photographic record showing negativity produced by KCl (all solutions at pH 8 2) indicating that the mobility of K^+ is greater than that of Na^+. The record starts with the cell in sea water. When the cell was transferred to sea water with 0 5 M KCl (p 24) the P D became 25 mv more negative. When the cell was replaced in sea water the P D began to fall. The vertical marks are 5 seconds apart. Temperature 22 C. Regarding Γ see Fig 1 (p 14)

was replaced in sea water. Then 0 01 M HG was added to the sea water with 0 5 M KCl and less negativity resulted (3) and when this was diluted (4) with 2 volumes of 8 7 per cent glycerol (containing 0 01 M HG) the P D became 12 mv more positive indicating that U_K was greater than V_{Cl}. At (5) the cell was replaced in 0 01 M HG in 'sea water with 0 5 M KCl' and left for 4 5 minutes after which (6) the dilute solution made the P D more negative by 6 mv, indicating that U_K had become smaller than V_{Cl} which is the reverse of the condition obtaining before the long exposure to HG[19]. As shown in Table II the average change due to dilution after treatment with HG was 12 9 mv (dilute solution negative)

Zero

25 mv

Zero

Zero

Zero

S.W.

S.W. with 0.5 M KCl

1

2

3 0.01 M HG

S.W. with 0.5 M KCl

Same +2 vol. glycerol

0.01 M HG + S.W. with 0.5 M KCl

F F

4

5

0.01 M HG + S.W. with 0.5 M KCl

Same + 2 vol. glycerol

F

6

0.01 M HG + S.W. with 0.5 M KCl

F

7

Fig 9

28
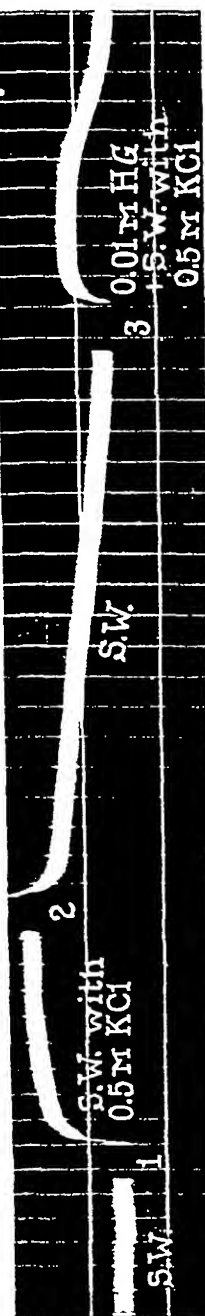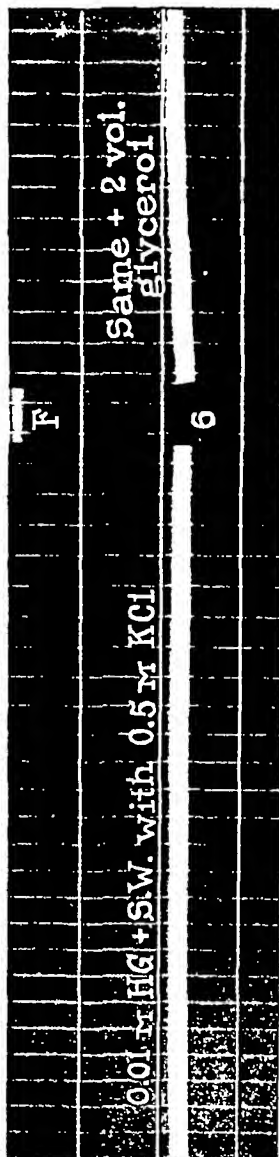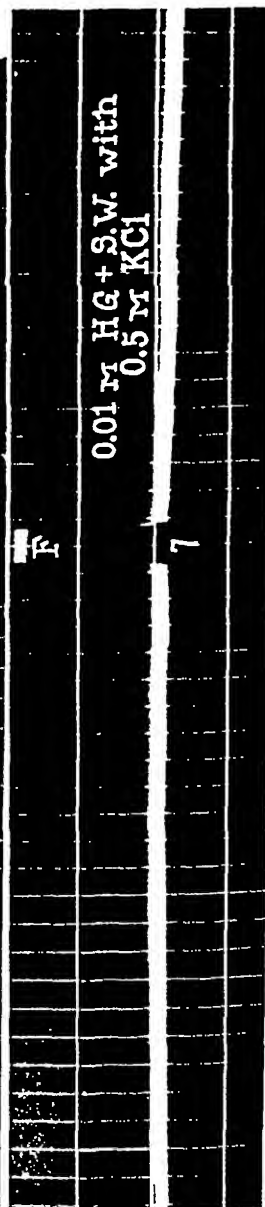
It is probable that the change is chiefly in U_K rather than in I_{Cl} because if V_{Cl} increased greatly it does not seem likely that U_{Na} could simultaneously become greater than I_{Cl} as described in the preceding section[6]

A similar falling off in U_K is seen when 0 6 M KCl is employed in place of "sea water with 0 5 M KCl." Dilution of 0 6 M KCl with 1 volume of glycerol (8 7 per cent) made the P D 8 2 mv more negative (See Table II)

This falling off in U_K may be responsible in part for the fact that when we add HG to "sea water with 0 5 M KCl" we get less negativity as is evident in Figs 9 and 10   (This also occurs with 0 6 M KCl) The falling off in negativity is often greater than that shown in these figures

In view of this it is not surprising to find that after an exposure to sea water plus 0 01 M HG of 3 minutes or more, "sea water with 0 5 M KCl" (p 24) can produce a positive[19] instead of a negative effect

Fig 9 Photographic record showing reversal of the concentration effect of KCl after exposure to HG (all solutions at pH 8 2)   At the start the cell was in sea water at (1) the cell was transferred to sea water with 0 5 M KCl (p 24) making the P D about 35 mv more negative at (2) it was replaced in sea water and the P D returned to the initial value at (3) it was placed in sea water with 0 5 M KCl' + 0 01 M HG making the P D more negative but to a smaller extent than before HG was applied this being followed by increased positivity due to HG (see Fig 10)   When this solution was diluted at (4), with 2 volumes of 8 7 per cent glycerol containing 0 01 M HC the P D became more positive by 11 8 mv, indicating that the apparent mobility of K^+ was greater than that of Cl^- but this behavior was reversed after a longer exposure to HG which began at (5) During this exposure which was made in 0 01 M HG + sea water with 0 5 M KCl the P D became more negative   At (6) this was diluted with 2 volumes of 8 7 per cent glycerol containing 0 01 M HG the P D now became 6 mv more negative (instead of more positive as happened previously) thus indicating that the apparent mobility of K^+ had become less than that of Cl^-, this was confirmed by replacing the cell at (7) in 0 01 M HG + "sea water with 0 5 M KCl upon which the P D became 6 mv more positive   The cell was subsequently returned to sea water (not shown in the record) and did not appear to be injured

The vertical marks are 5 seconds apart   Temperature 21 C   Regarding Γ see Fig 1 (p 14)

[6] This change in U_{Na} would tend to make the dilute solution positive but this is masked by the greater change occurring in U_K in the opposite direction
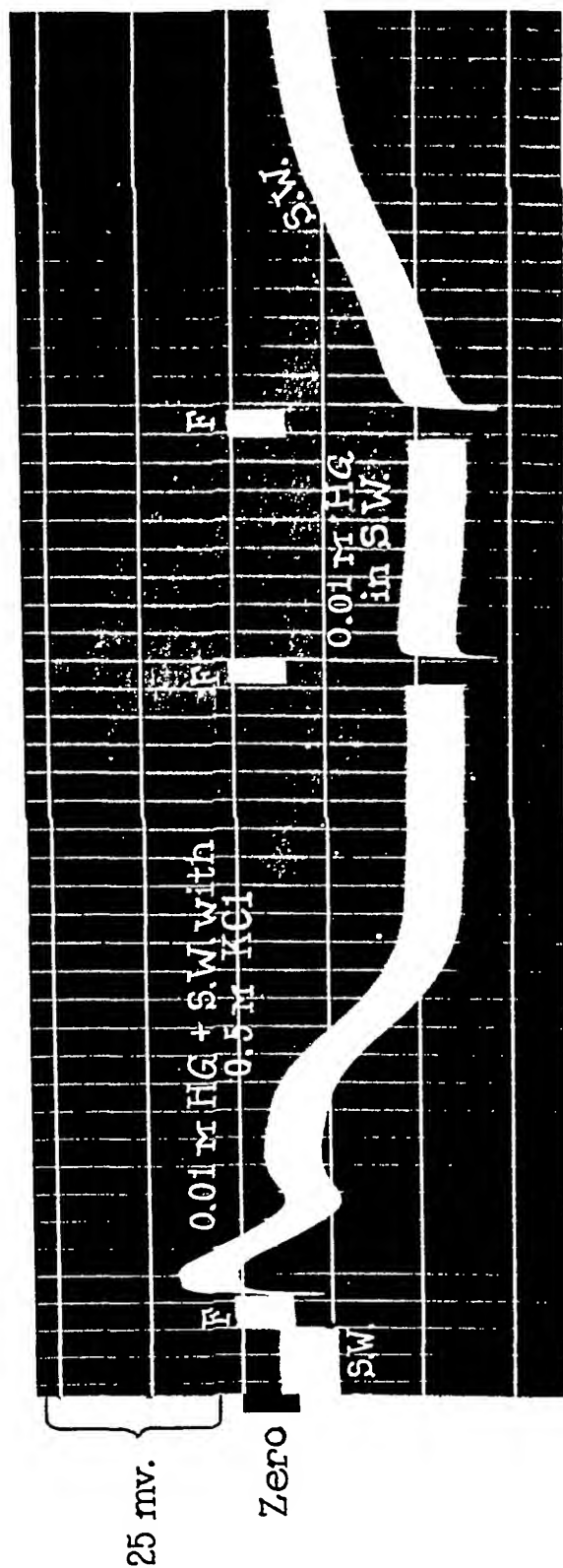
FIG. 10. Photographic record showing that KCl produces less negativity when 0.01 M HG is present (compare with Fig. 8). All solutions at pH 8.2. The cell was in sea water at the start and was then transferred to 0.01 M HG in "sea water with 0.5 M KCl" (p. 24), in which it became 18 mv. more negative. After this the curve fell. On transferring to 0.01 M HG in sea water there was practically no change, indicating that the apparent mobility of K⁺ had become approximately equal to that of Na⁺. In sea water the P.D. went back approximately to the original value.

The time marks are 5 seconds apart. Temperature 23°C.

Regarding F see Fig. 1 (p. 14)

30
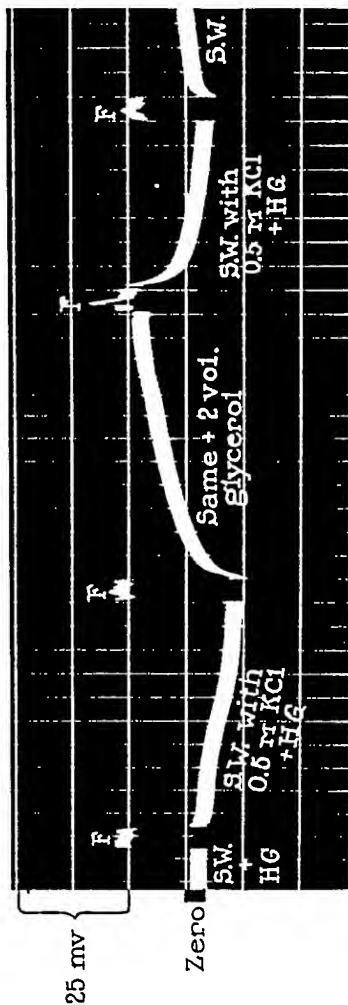
FIG 11 Photographic record showing the reversal of the action of KCl after an exposure of 335 seconds to 0 01 M HG in sea water On changing from sea water + 0 01 M HG to sea water with 0 5 M KCl (p 24) (containing 0 01 M HG) the p D became 7 7 mv more positive (instead of more negative) On diluting this with 2 volumes of 8 7 per cent glycerol (containing 0 01 M HG) the p D became more negative (instead of more positive) by 20 2 mv The cell was then returned to 'sea water with 0 5 M KCl + 0 01 M HCl and then to sea water in which the p D came back approximately to the original value it had at the start of the experiment

The time marks are 5 seconds apart Temperature 23 C Regarding Γ see Fig 1 (p 14)

31
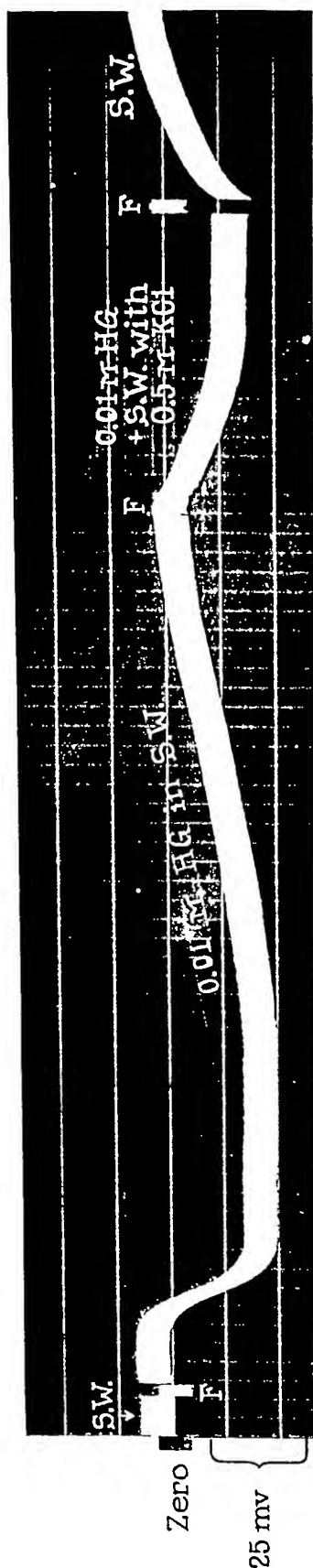
FIG 12 Photographic record showing that after sufficient exposure to HG the change produced by "sea water with 0 5 M KCl" is positive instead of negative (compare with Figs 8, 9, and 10)   All solutions at pH 8 2   The cell was at first in sea water it was transferred to 0 01 M HG in sea water   After a latent period of about 16 seconds there was a positive drop of about 28 mv followed by recovery, the total exposure to HG being 230 seconds   The cell was then placed in 0 01 M HG in "sea water with 0 5 M KCl" (p 24) which instead of producing negativity, as happens when there has been no previous exposure to HG (or a short exposure as in Fig 9), made the P D more positive by 17 mv, indicating that the apparent mobility of K+ had become less than that of Na+   The cell was then replaced in sea water and the P D returned to the value it had at the start After a rest of 4 hours in sea water it was placed in "sea water with 0 5 M KCl" (not shown on the record) which made the P D strongly negative, in dicating that the apparent mobility of K+ had become much greater than that of Na+

The time marks are 5 seconds apart   Temperature 22°C   Regarding F see Fig 1 (p 14)

(Figs 11 and 12), thus indicating that $U_K < U_{Na}$. As shown in Table II, the average change was 20 4 mv more positive

Putting $V_{Cl} = 1$, Damon[1] found U_K to be 20 but states that there was considerable variation. The cells used in the present investigation showed great variation, the value of U_K ranging from about 5 to 20. The value of U_K after treatment was calculated by using the formula given on p 23 from the I D between 0 6 M and 0 3 M KCl which was 8 2 mv (Table II), we get $U_1 = 0.36$

Experiments with Potassium Guaiacolate[17]

Since K^+ has a high apparent mobility and in some cases this applies also to G^-, it seemed desirable to add both together. Accordingly experiments were made by adding potassium guaiacolate to the sea water at pH 8 2. The results, as seen in Table I, were similar to those obtained by adding HG

These experiments indicate that a profound alteration of the properties of the protoplasmic surface may be brought about by undissociated guaiacol (since at pH 6 4 the concentration of G^- is negligible)

DISCUSSION

It has been found in previous work that the most satisfactory way to deal with such P D measurements is to regard them as chiefly due to diffusion potentials[1,16]. Let us now consider the behavior of potassium from this point of view

The Potassium Ion —When *Valonia* is transferred from sea water to "sea water with 0 5 M KCl" (p 24) there is a marked increase in negativity[1]. When this solution is diluted with an equal volume of isotonic glycerol the P D becomes more positive. Since the more dilute solution is more positive we conclude that $U_K > V_{Cl}$. When the more dilute solution of KCl becomes more negative after the guaiacol treatment we may conclude that $V_{Cl} > U_K$. Whether this is due to a falling off in U_K or to an increase in I $_{Cl}$ cannot be decided but if the latter increased much it might not be possible for U_{Na} to become greater than I $_{Cl}$, as apparently happens

Charged Complexes —These results are not in harmony with Walden's rule which states that the ratio $U_K - U_{Na}$ should be approximately the same in all solvents

If the guaiacol treatment caused K^+ to become more solvated it

would lower the value of U_K but it does not seem probable that the observed changes could be accounted for on this basis [27]

If under normal conditions the partition coefficient[28] of KCl exceeded that of NaCl and U_K was equal to U_{Na} our calculations would make it appear that U_K exceeded U_{Na} when the values were deduced from the "chemical effect" (i e the negativity produced by substituting KCl for NaCl), but not when deduced from the "concentration effect" (i e the change produced by diluting a solution of KCl or NaCl)   But if it happened that the partition coefficient of KCl increased more rapidly with concentration than that of NaCl we should deduce too high a value of U_K from the concentration effect also

Some changes in activity partition coefficients may occur when alterations take place in one or both of the phases concerned, but changes of great magnitude are improbable   On the other hand it seems possible that the partition coefficient of K^+ might appear to increase more than that of Na^+ by the formation of charged complexes which are found, according to Kraus,[29] in media of low dielectric constant such as we suppose the non-aqueous surface layer of the protoplasm to be [30]   Thus if we have K^+ in the external solution we might have in the non-aqueous layer K^+, $(KX_I)^+$, $(KX_{II})^+$, etc, all of which would count in our calculations as K^+ (X_I and X_{II} may be elements or radicals) and thus increase the apparent ionic partition coefficient

[27] Shedlovsky, T, and Uhlig, H H, *J Gen Physiol*, 1933–34, **17**, 563   It should be said, however, that small differences may be due to solvation   Thus Kraus explains the order $U_{Rb} > U_{Cs}$ in liquid ammonia as due to solvation (in water $U_{Cs} > U_{Rb}$)   It is of interest to note that we find $U_{Rb} > U_{Cs}$ in *Nitella* and in *Valonia* (unpublished results of E B Damon), but here the differences are much greater than in liquid ammonia

[28] This becomes evident from the following considerations   In the equation given on p 23 the actual value of the partition coefficient does not matter since only the ratio $(C_1 - C_2)$ counts and this will remain the same regardless of the value of the partition coefficient so long as the latter is the same for both concentrations   But if the partition coefficient increases with concentration so that the ratio $(C_1 - C_2)$ increases the calculated value of U_K will increase

For the chemical effect we employ Henderson's equation in which the actual concentrations become important and an increase in the apparent partition coefficient (due to formation of charged complexes) affects the result

[29] Kraus, C A, *Tr Electrochem Soc*, 1934, **66**, 179

[30] Osterhout, W J V, *Ergebn Physiol*, 1933, **35**, 1003

This apparent increase might be greater for K^+ than for Na^+, thus changing the calculated ratio $U_K - U_{Na}$. It seems possible that HG might act by changing such charged complexes

This might also apply to the formation of chemical compounds in the non aqueous layer. If for convenience in discussion we use the same standard state for both phases, confining our attention to the thin layers on each side of the phase boundary which are assumed to be practically in equilibrium we may say that the ionic activity product $[K] [Cl]$ is the same in both. But if in the non aqueous layer organic potassium compounds are formed so that we have not only KCl but KR, KR_{II}, etc, which are not very soluble in water the activity of K^+ may be very much greater in the non aqueous although the product $[K] [Cl]$ remains the same. Thus if we have for activities in the external solution $K^+ = 0.01$ and $Cl^- = 0.01$ we may have in the non aqueous phase $K^+ = 0.1$ and $Cl^- = 0.001$

But if in the external solution the product $[Na] [Cl]$ equals $[K]$ $[Cl]$ we should expect this to hold also for the non aqueous phase since in the latter $Cl^- = 0.001$ we expect $Na^+ = 0.01 = K^+$. We thus have no difference between Na^+ and K^+ (but the dissociation constants and the undissociated molecules may not have the same values in both cases)

Although such assumptions might explain certain variations in the ratio $U_K - U_{Na}$ it is not clear that they can account for the fact that after exposure to HG we get $U_{Na} > V > U_K$ to produce this result actual changes in mobilities may be necessary

For convenience we may hereafter speak of U_K with the understanding that its value may depend in part on complexes or on chemical combination. This applies also to U_{Na}

Not much can be said about the nature of these charged complexes or compounds at present but it may be recalled that there are salts of sodium and of potassium which are said to be more soluble in non polar solvents than in water. (Calcium salts of this sort have been isolated from plants[2] and calcium oleate is more soluble in certain non aqueous solvents than in water[3])

[1] Hundeshagen F J prakt Chem 1883 N s 28, 219
[2] Smith J A B and Chibnall A C and Chibnall A C and Channon N J Biochem J London 1932 26, 1345 Biochem J London 1929 23, 176
[3] Experiments in this laboratory by J W Murray showed that when a large

Chemical reaction in the non-aqueous protoplasmic surface appears to occur when ammonia[34] and guanidine[35] enter *Valonia* and may be characteristic of bases generally apparently this does not apply to acids [36] To what extent Cl^- enters into the complexes may be left open for the present   In this connection it may be recalled that guaiacol forms not only salts but esters, some of the latter are said to be insoluble in water, *e g* , guaiacol oleate   It is possible that compounds of this sort may be formed in the protoplasmic surface

It is probable that *p*-cresol and phenol,[37] which are chemically related to guaiacol, will act like guaiacol since they affect the P D in somewhat the same way as guaiacol when added to sea water at pH 8 2   But this is not true of such acids as formic, acetic, valeric, benzoic, and salicylic

It may be added that exposure of *Nitella*[38] to distilled water (which dissolves organic substances out of the cell) changes the order of apparent mobilities from K > Na to Na > K   Here also charged complexes may be involved

Behavior of the Outer Protoplasmic Surface —It might be suggested that HG puts the outer surface out of commission[39] so that the observed

volume of an aqueous solution of 0 0012 M Ca(OH)$_2$ was shaken with a little iso-amyl alcohol containing 0 1 M oleic acid the proportion of Ca^{++} in the alcohol to that in the aqueous solution was as 16 8 to 1 (temperature about 25°C)   See also Bancroft, W D , *J Phys Chem* , 1913, **17**, 501, Clowes, G H A , *J Phys Chem* , 1916, **20**, 667   According to Seidell, A , Solubilities of inorganic and organic compounds, New York, D Van Nostrand Co , 2nd edition, 1919, p 209, calcium oleate is much more soluble in glycerol than in water

[34] Osterhout, W J V , *Proc Nat Acad Sc* , 1935, **21**, 125
[35] Jacques, A G , *Proc Nat Acad Sc* , 1935, **21**, 488
[36] Jacques, A G , *J Gen Physiol* , 1935–36, **19**, 397

[37]

Phenol   *p*-cresol   Guaiacol
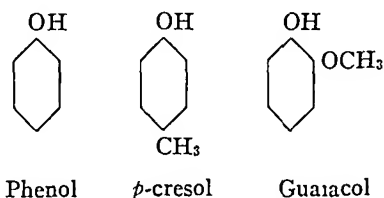
[38] Osterhout, W J V , *J Gen Physiol* , 1934–35, **18**, 987
[39] *E g* by breaking down its structure or by increasing its permeability to all the ions concerned so that their apparent mobilities became nearly the same

I D s are those of the inner surface where the order of apparent
mobilities may be quite different If this were the case, sudden
changes in the external solution could not produce sudden changes
in I D since time would be required for the substituting ions to diffuse
across the protoplasm to the inner surface We find, however, that
external changes continue to produce as rapid changes in P D (often
beginning within 1 or 2 seconds)[40] as before This applies to changes
in the concentration[41] of sea water (Fig 6), of KCl (Figs 9 and 11)
and of NaCl (Fig 5), as well as to the change from sea water to 0 6
M KCl (Fig 12)

Moreover, Blinks[4] finds that p cresol (which is chemically close to
guaiacol) does not tend to destroy the outer surface but rather to
conserve it

The Sodium Ion –In NaCl the more dilute solution is normally
more negative because U_{Na} is less than I_{Cl} but after exposure to
guaiacol the more dilute solution may become the more positive
indicating that U_{Na} has become greater than I_{Cl}[43] (Fig 5)

Since the concentration effect of sea water is essentially that of
NaCl[1] it is not surprising that it can be reversed by guaiacol (Fig 7)
just as in the case of NaCl

The Guaiacol Curve a The Positive Drop —The curve shown in

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[40] It seems extraordinary that the change is so rapid when the protoplasm is
covered with a cellulose wall which may be as much as 10 microns thick It is
possible that there are protoplasmic processes passing more or less completely
through the cellulose wall The change in U_K and U_{Na} which occurs in the outer
surface layer requires an exposure to HG of 5 minutes to bring about the reason
for this is not known

[41] The change in P D due to change in liquid junction as the result of change in
concentration is of course instantaneous but this is so small as to be neg-
ligible

With dead cells the change of P D was negligible[18] If it were large enough to
appear on the record we should see at the start a sudden movement of the curve
followed by an abrupt change in its course in case the alteration in the P D of the
cell were relatively slow As nothing of the sort is observed we may conclude that
the P D of the cell changes rapidly from the start.

[4] Blinks L R *J Gen Physiol*, 1935–36 19, 633

[43] In some cases transfer to the dilute solution does not change the P D, here
U_{Na} appears to be equal to I_{Cl}

Fig 1 can be obtained in 0 6 m NaCl[44]   We then have at the outer surface only Na^+ and Cl^- to consider   We have seen that HG increases the apparent mobility of Na^+ as compared with Cl^- so that the addition of HG to NaCl will tend to make the P D at the outer surface more negative   The increased positivity must therefore depend on other ions

It seems probable that these are chiefly the organic[45] ions in the protoplasm which have been mentioned in previous papers[46]   They may be responsible for the normally negative P D   for the inorganic ions of the sap and sea water would presumably produce a positive P D   The sap contains 0 5 m KCl + 0 1 m NaCl, which should produce an outwardly directed P D   if the inner surface layer of the protoplasm (which we may call Y) has properties[47] at all resembling those of the outer surface layer (which we may call X)   The action of the sea water on X would also produce an outwardly directed positive potential since $U_{Na} < V_{Cl}$ and the effect of the other ions of sea water is small

Between X and Y lies the main bulk of the protoplasm, forming an aqueous layer which we may call II[48]   We may suppose that organic ions in this layer are responsible for the fact that the normal P D

[44] The fact that a greater positive drop is obtained with 0 6 m NaCl + HG than with sea water + HG (Table I) may mean that the surface is affected differently in the two cases but in the absence of HG no difference is observable in such short experiments

[45] Little or no calcium, magnesium or sulfate passes through the protoplasm into the sap   Cf Osterhout, W J V , Ergebn Physiol , 1933, 35, 981

[46] Osterhout, W J V , Bull Nat Research Council, No 69, 1929, 170, footnote 65, Biol Rev , 1931, 6, 382   Osterhout, W J V , and Hill, S E , J Gen Physiol , 1934–35, 18, 499

[47] Blinks has found evidence (Blinks, L R , J Gen Physiol , 1934–35, 18, 409) which he regards as somewhat doubtful, indicating that K^+ does not greatly affect the P D of Y The experiments were made by piercing the vacuole with two capillaries, one for the entrance and the other for the exit of a perfusing solution   The cell does not live well after this treatment and the capillaries are as a rule soon plugged with a gelatinous plug which stops perfusion (this is not the case with *Halicystis* where perfusion succeeds beautifully and the cell continues to live)   When sea water was perfused in the vacuole there was not much change in P D (sea water contains 0 012 m K^+ and sap about 0 5 m K^+)

[48] Osterhout, W J V , Ergebn Physiol , 1933, 35, 1013

is about 10 m\ inwardly directed This can only be possible when
\ and J have different properties that this is the case is shown by
placing sap outside \ so that we have the chain

<div align="center">

protoplasm

Sap outside $\lceil$ \ $\mid$ H $\mid$ I $\rceil$ sap inside

</div>
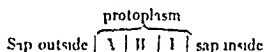
This gives about² 63 m\ showing that \ and J are unlike

It therefore seems necessary to assume that organic ions in H can
produce an appreciable i p by having unlike mobilities in \ and J
If HG can alter the apparent mobilities of these ions (as it does those
of Na⁺ and K⁺ in \) this may explain the guaiacol curve

That the positive movement of this curve does not depend primarily
on changes in the inorganic ions is evident because the normal apparent
mobilities of K⁺, Cl⁻, and Na⁺ persist for some time after the
positive drop of the curve as shown by the concentration effects
Why this is so is not clear Perhaps the simplest suggestion is that
a gradual organic reaction gives a compound which affects appar-
ent mobilities according to its concentration, a higher concentration
being required in the case of K⁺, Na⁺ and Cl⁻ (whose apparent mo-
bilities change gradually as would be expected on this basis)

b The Threshold and Latent Period — Apparently there is a critical
concentration below which HG has little or no effect and the latent
period is presumably the time required for HG to diffuse into \ and
reach this concentration Hence the latent period shortens as the
external concentration of HG increases (p 17) (The latent period
will lengthen if chemical transformation in the protoplasm renders
part of the HG ineffective)

As might be expected we find practically no latent period when 0 01
m HG is applied to a cell in contact with 0 005 m or 0 0075 m HG which
has produced no response For in this case the concentration of HG
in \ is already near the critical value before 0 01 m HG is applied [49]

One possible explanation of the threshold is suggested by the study
of models in which HG is used to represent the non aqueous layer of
the protoplasmic surface HG is employed because in many respects

[49] With phenol we find something resembling a latent period where none is
found with guaiacol Possibly phenol penetrates more slowly (with a smaller ionic
radius its partition coefficient may be smaller ⁷)

it behaves like the protoplasm of *Valonia*[50] but it is not probable that the non-aqueous surface layer of *Valonia* is homogeneous any more than it is in *Nitella*[4] and as a matter of fact we can imitate the behavior of *Valonia* more closely by mixing HG with certain other substances

We can, for example, cause the rate of entrance of potassium to fall off and thus to approach that observed in *Valonia* by mixing chloroform with HG   At the same time the rate of entrance of the basic dye brilliant cresyl blue at low pH falls off and becomes more like that with *Nitella*[51] and *Valonia* as described by Irwin

Following up this suggestion we may imagine a model with a non-aqueous layer consisting of a mixture of organic solvents previously shaken with sea water containing 0 005 M HG   On placing this in contact with sea water we assume that HG goes out very slowly If we add 0 005 M HG to the sea water none will be taken up but if we increase HG in the sea water to 0 01 M its concentration in the non-aqueous layer will at once increase   If this increase should raise the value of U_{Na} and lower that of U_K sufficiently we might get effects like those seen in *Valonia*

This suggestion encounters difficulties when we consider other substances   For example, phenol and *p*-cresol have much the same action as HG on *Valonia*   How are we to deal with these?

If the protoplasmic surface contains HG it must be formed in the cell and slowly pass out (as does R in *Nitella*[4])   That its exit would be gradual is indicated by the fact that after an exposure of 2 minutes to 0 01 M HG it may require several hours to wash it out and restore the cell to its original condition   With 0 005 M HG added to the sea water this exit would presumably stop but the concentration of HG in the non-aqueous layer would increase very slowly since its production must be very slow to keep pace with its exit under normal conditions   But if the concentration of HG in the sea water is sufficiently increased it must increase in the protoplasmic surface

When we place 0 005 M phenol in the sea water and obtain no more response than with 0 005 M HG it becomes necessary to suppose that the surface already contains phenol or that the phenol is ineffective for some other reason   For the phenol in the protoplasmic surface is now added to the guaiacol already present

[50] Osterhout, W J V , and Stanley, W M , *J Gen Physiol*, 1931–32, **15**, 667
[51] Irwin, M , *J Gen Physiol*, 1922–23, **5**, 727, 1925–26, **9**, 561

A similar difficulty arises in general, whenever a variety of penetrating substances exhibit thresholds   But when substances produce their effects by dissolving out materials from the cell (as distilled water does in *Nitella*[4]) this difficulty does not arise because the threshold will be passed whenever the material is dissolved out more rapidly than it is produced in the cell

It would therefore seem that the question of the threshold must be left open

c   The "Recovery" — After the fall of the curve under the influence of 0 01 M HG it may return to its original value   For convenience this is called "recovery" although this term is not meant to imply that the cell returns to its original state   This behavior is understandable if the inwardly diffusing HG reaches J and there produces the same effect as in A for the change in P D in A would then be cancelled by an equal and opposite change in P D in J (owing to the fact that the organic ions are diffusing inward[5] through J and outward through A)

Possibly the apparent mobilities of Na and K in Y are also changed in such fashion as to aid recovery   Changes in acidity seem to be of little importance since a variety of organic acids failed to produce any effect (see p 42)

The rise of the curve would, of course, be slower than the fall because of the time required for HG to diffuse across A and W (H is presumably much thicker than A or Y) to reach J   The form of the curve indicates that there is not much latent period for J (perhaps because J has a lower threshold)

E B Damon[53] suggests that the positive drop might be due to increased permeability of Y, letting K^+ move outward from the sap (which contains about 0 5 M KCl) until it comes in contact with the inner surface of A   But it is questionable whether the inward diffusion of HG and the subsequent outward diffusion of K^+ could take place within the short latent period of 15 to 25 seconds   Moreover, it is

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[5] It might be supposed that these ions would reach the same concentration in the sap as in H so that inward diffusion would cease   But this would not be apt to happen in growing cells and even in cells which had ceased to grow these ions might be chemically combined in the sap   In the absence of facts no conclusion can be reached

[53] Private communication

not clear how the behavior of the curve during recovery could be accounted for   This scheme would not explain the reversal of the concentration effect with KCl, NaCl, and sea water

When the cell is replaced in sea water during or after recovery in HG it is possible that HG may diffuse out of X somewhat faster than out of Y This would tend to make the P D temporarily more negative   it is seen occasionally but as a rule is not perceptible

These effects are apparently not due to acidity alone for phenol, p-cresol, and p-amino phenol (chemically related to guaiacol) are the only organic acids thus far studied which appear to act like HG in our experiments   The following were tested in sea water[54] at pH 8 2 (at concentrations of 0 001, 0 005, 0 01, 0 02, 0 04, and 0 08 M)   formic,[55] acetic, valeric,[55] benzoic, and salicylic   They had practically no effect on the P D

Variability —The cells showed great variability   their behavior appeared to depend somewhat on the length of time after impalement and on external conditions   Perhaps this variability is related in some cases to the "stages" described by L R Blinks [45]   In some cases no response was obtained[20] on adding HG (up to 0 03 M) to sea water at pH 8 2 or at 6 4   Evidently the outer protoplasmic surface is subject to considerable variation in composition   This is in line with the variation observed in applying KCl to *Valonia*[1] and in the concentration effect of sea water[21] with *Valonia*, and of KCl[16] and NH_4Cl[56] with *Nitella*

In view of this variability it would seem that the protoplasmic surface can hardly be a monomolecular layer [57]   If it is a thicker layer it may have interesting possibilities[58] of variation   It seems possible that great changes in the composition of such a layer might result from metabolism so that the apparent ionic mobilities might show con-

[54] The acid was added to the sea water and NaOH was then added to produce a pH of 8 2

[55] 0 08 M was omitted

[56] Unpublished results

[57] *Cf* Osterhout, W J \ , *Physiol Rev*, 1936, **16**, 216

[58] Teorell (Teorell, T , *Science*, 1935, **81**, 491) has shown that a diffusion potential across a membrane can produce great inequalities in the distribution of ions in the membrane   This distribution would change with changes of relative mobility and concentration of ions in the solutions in contact with the membrane

siderable variation   It is also possible that physical factors (especially radiation) and chemical influences in the environment may act similarly

The foregoing discussion assumes that diffusion potentials play the chief rôle, a procedure which has proven useful in several cases [1] [16] To what extent other potentials, especially phase boundary potentials [59] enter in is an open question but it is difficult to see how phase boundary potentials can account for the fact that HG causes U_{Na} to increase and U_K to decrease

<div align="center">SUMMARY</div>

In normal cells of *Valonia* the order of the apparent mobilities of the ions in the non aqueous protoplasmic surface is $K > Cl > Na$ After treatment with 0 01 M guaiacol (which does not injure the cell) the order becomes $Na > Cl > K$

As it does not seem probable that such a reversal could occur with simple ions we may assume provisionally that in the protoplasmic surface we have to do with charged complexes of the type $(KX_I)^+$, $(KX_{II})^+$, where X_I and X_{II} are elements or radicals, or with chemical compounds formed in the protoplasm

When 0 01 M guaiacol is added to sea water or to 0 6 M NaCl (both at pH 6 4, where the concentration of the guaiacol ion is negligible) the P D of the cell changes (after a short latent period) from about 10 mv negative to about 28 mv positive and then slowly returns approximately to its original value (Fig 1, p 14)   This appears to depend chiefly on changes in the apparent mobilities of organic ions in the protoplasm

The protoplasmic surface is capable of so much change that it does not seem probable that it is a monomolecular layer   It does not behave like a collodion nor a protein film since the apparent mobility of Na^+ can increase while that of K^+ is decreasing under the influence of guaiacol

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[59] For the combined effect of boundary' potentials and diffusion potentials see Teorell T , *Proc Soc Exp Biol and Med* , 1935-36 **33**, 282

PIGMENTS OF THE RETINA

II SEA ROBIN, SEA BASS, AND SCUP

By GEORGE WALD

(From the Woods Hole Oceanographic Institution, Woods Hole,* and the Biological Laboratories of Harvard University, Cambridge)

In the preceding paper of this series (Wald, 1935–36 b) the visual purple system of the bull frog was found to conform in part with the equations
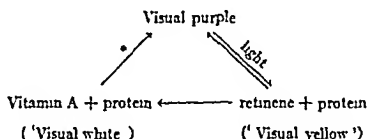
in which all but the reaction starred occur in the isolated retina as well as in the intact eye

Identical processes are found in the retinas of the sea robin (*Prionotus carolinus*), the black sea bass (*Centropristes striatus*), and the scup or porgy (*Stenotomus chrysops*) In the pigment epithelium and choroid layer of these fishes pigments occur also which are either identical with or very closely related to those in frogs

Kottgen and Abelsdorff (1896) found the absorption spectrum of visual purple from amphibia, owls, and mammals to possess a maximum at about 500 mμ, while that from eight species of fresh water fishes, though of the same general form, is displaced so that its maximum occurs at about 540 mμ This spectral peculiarity, if general, should lend special interest to an examination of the visual system in

45

fishes   However, visual purple solutions from the three marine species discussed in the present paper have been found to be almost identical spectroscopically with those of frogs, the maxima occurring in each case at about 500 mμ The apparent discrepancy between these and Kottgen and Abelsdorff's results is being investigated further

Retinas

Experiments performed in the examination of frog retinas have been repeated with fish tissues with identical results   The dark adapted retinas contain a small quantity of free vitamin A and a large amount of bound retinene   The latter is liberated by destroying the visual purple, either with light or with chloroform   Retinene liberated by light is subsequently converted to vitamin A by a thermal reaction, evidenced by the fading of the visual yellow retina to colorlessness

I shall not describe the details of these experiments again, but instead record a simple procedure which has served to establish the nature of the visual purple system in a single experiment   To follow this one need only recall that both retinene and vitamin A yield blue colorations when mixed with antimony trichloride reagent, due in the case of retinene to an absorption band at 662–666 mμ, in that of vitamin A to one at 612–615 mμ

Figs 1 and 2 show the results of this type of experiment performed with scup and sea robin retinas, Fig 3 a slight variant of this procedure with bass tissues [1]

Right and left retinas of five dark adapted fishes (scup and sea robin, 8 to 10 inches long) were prepared separately   One set of five retinas was extracted thoroughly in the dark with about 12 cc of benzine in 4 portions, shaking violently by machine for a total of 20 minutes   The extract brought into chloroform was colorless and when tested with antimony trichloride yielded blue solutions of which Curves *a* of Figs 1 and 2 are the absorption spectra   The dark adapted retina therefore contains a small quantity of vitamin A alone

The same tissues were bleached subsequently in daylight to an orange color ("visual yellow") and were immediately re-extracted

with benzine The extract, brought into chloroform, was bright
yellow Tested with antimony trichloride it yielded Curves b of Figs
1 and 2 The bleaching of visual purple to yellow therefore liberates
a large quantity of retinene

FIG 1 Absorption spectra of the antimony trichloride reaction with benzine
extracts of scup retinas a, Dark adapted retinas, the extract contains a small
quantity of vitamin A (615 mμ chromogen) b, The same retinas, re-extracted
immediately after bleaching A large quantity of retinene (664 mμ chromogen)
has been liberated c, Retinas from the same fishes extracted 1 hour after
bleaching The free retinene has been converted to vitamin A

The second set of five retinas was bleached in daylight and left at
room temperature in moderate light for an hour During this period
the retinas faded from orange to colorlessness They were extracted
with benzine exactly as before The extract, brought into chloroform,

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[1] All spectra shown in this paper were recorded automatically with a photoelectric spectrophotometer (Hardy, A C , *J Opt Soc America*, 1935, **25**, 305) at the Massachusetts Institute of Technology

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room temperature in moderate light for an hour   During this period
the retinas faded from orange to colorlessness   They were extracted
with benzine exactly as before   The extract, brought into chloroform,

was colorless, and when tested with antimony trichloride yielded Curves *c* of Figs 1 and 2   The fading process converts retinene liberated in bleaching quantitatively to vitamin A
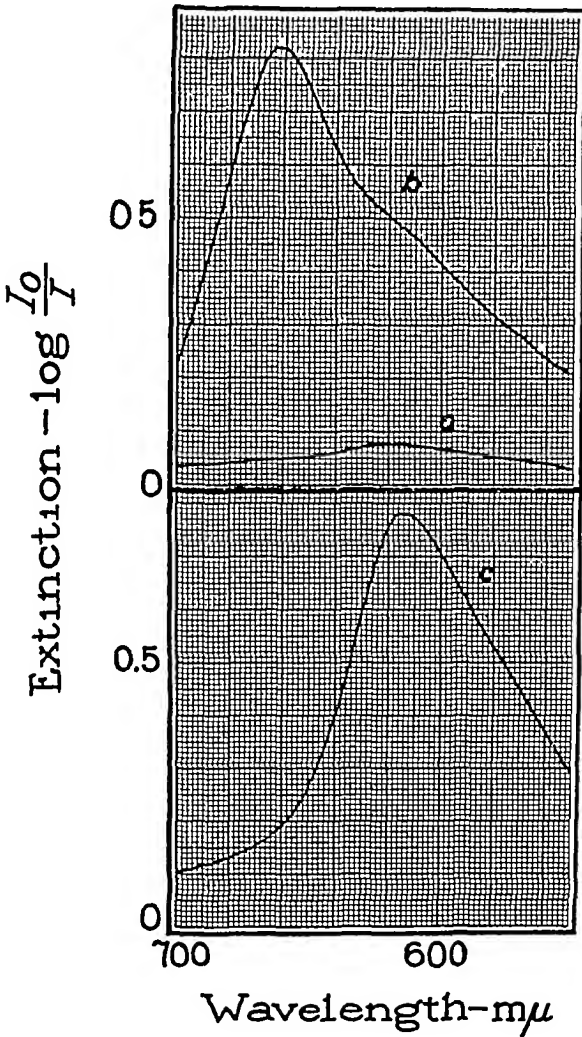
FIG 2 Absorption spectra of the antimony trichloride reaction with benzine extracts of sea robin retinas   Compare with Fig 1

At the time of these experiments only 3 bass about 14 inches long were available   The retinas of two of these were used to prepare a visual purple extract   The residual tissue from this procedure still contained visual purple, which was destroyed by extracting with

chloroform   The extract was yellow, and with antimony trichloride
yielded the upper curve of Fig 3, showing the presence of retinene
and a small amount of vitamin A   The retinas from the single re-
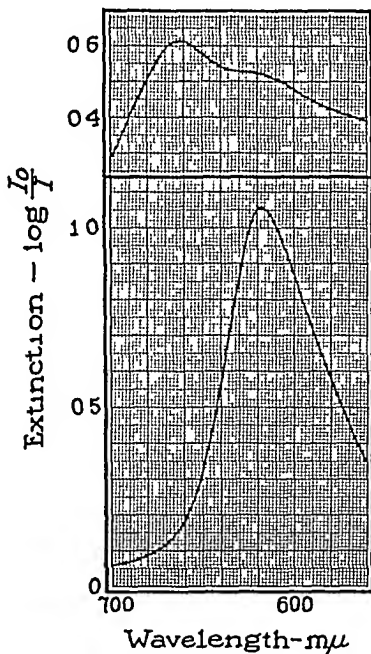
FIG 3 Absorption spectra of the antimony trichloride reaction with extracts
of sea bass retinas   *Upper curve* chloroform extract of dark adapted retinas
Lower curve, benzine extract of bleached and faded retinas

maining fish were bleached in daylight and allowed to fade for an hour
They were then extracted with benzine   The colorless extract, tested
with antimony trichloride, yielded the lower curve of Fig 3, showing,

as in the other forms, the conversion of retinene liberated by light to vitamin A

Maximal quantities of vitamin A occur in retinas which have been bleached and allowed to fade completely *in vitro*   These quantities, estimated roughly by a colorimetric method (Wald, 1935–36 *a*) are sea robin, 1 4, scup, 2 8, and bass, 4 7 γ per retina   The retinal dimensions in the specimens used increased in the same order, so that it is possible that the quantity of vitamin A per unit weight of retina is approximately constant in all three species

Acid-Base Effects upon the Visual Yellow Retina —Characteristic responses of the visual yellow retina to acids and alkalies have been observed in frogs and fishes and examined in some detail in the sea robin   Chase (1935–36) has shown that a yellow product of the bleaching of visual purple in solution behaves as an acid-base indicator, turning colorless in alkaline solutions   This observation is closely related to those to be described   The behavior of the retinal pigments *in situ*, however, is much more complicated than in solution, and the precise connections between the two situations are still to be elucidated   At present certain features in the acid-base behavior of the sea robin retina may be indicated

The bleached, visual yellow retina is of a distinctly orange color   When made sufficiently alkaline, it turns practically colorless, if made strongly acid, bright yellow   The change from yellow to colorless is freely reversible, the tissue behaving as an acid-base indicator   However, I have never succeeded in bringing such retinas back to the original orange color of visual yellow   Some irreversible change therefore accompanies these abnormal pH's

The bright yellow, comparatively photostable material which is formed when the retina is treated with strong acids has long been employed as a test for visual purple (Boll, 1877, van Genderen Stort, 1887)   In reality it is a test for retinene or visual yellow   It is yielded by dark adapted and visual yellow retinas, but not by retinas which have been bleached and allowed to fade completely, that is, in which the retinene has been converted to vitamin A   Since the acid yellow color is considerably deeper than that of neutral visual yellow, it reveals the presence of retinene in retinas which when neutral may appear quite colorless   In this way the last traces of retinene in the fading retina may be distinguished

Neutral visual yellow retinas, cooled to 0°C., retain their color for hours, even in bright daylight. Such retinas may be titrated on ice with alkalies to determine the pH at which the color change occurs. In 0 035N NaOH or 0 35N NH₃ the orange retina loses its color within about 10 minutes at 0°C in daylight. In more strongly alkaline solutions or at higher temperatures the reaction is more rapid. These time intervals are not needed primarily for penetration of the reagent, for a dark adapted retina soaked in 0 23N NH₃ for ½ hour in the dark before being exposed to light on ice, first turns orange and then still requires about 12 minutes to fade to colorlessness. This period is apparently principally occupied by the irreversible reaction which transforms visual yellow to the yellow colorless pH indicator.

The pH at which the visual yellow retina is decolorized is well outside the physiological range. The concentrations of alkali cited above as approximately minimal correspond to pH's of about 11–12. In such solutions the tissue disintegrates rapidly. Dark adapted retinas, soaked for 10 minutes in phosphate buffers at pH 6 0 and 8 0 before being exposed to light, exhibited no detectable differences either in color before and after bleaching or in rates of bleaching, fading, and regeneration. A retina at pH 9 0 behaved similarly. The acid base changes discussed above are therefore decidedly abnormal.

In ammoniacal retinas (0 23N NH₃) the colorless derivatives of visual yellow are not removed to form vitamin A, nor do they regenerate visual purple in the dark. Even after 3 hours in the light at room temperature their presence is revealed by the strong yellow color which develops when the retina is acidified. On the other hand the reversion of visual yellow itself to purple is greatly accelerated in 0 23N NH₃. Ammoniacal retinas, returned to the dark immediately after bleaching, before the visual yellow has been appreciably decolorized by the alkali, regenerate considerable amounts of visual purple within ½ hour even at 0°C., though at this temperature neutral retinas do not change appreciably within a comparable period. Curiously, the ammoniacal visual yellow retina regenerates more visual purple at 0°C than at room temperature. It seems as though two reactions compete for the removal of visual yellow—reversion to purple, and the irreversible formation of the pH indicator, and that the former is relatively favored at low temperatures.

The accelerating effect of NH₃ upon the reversion process explains Kuhne's observation, which I have confirmed, that the ammoniacal retina bleaches more slowly than the neutral tissue (Kuhne, 1878) This difference cannot be ascribed to induced photostability in the visual purple itself, for ammoniacal visual purple *solutions*—in which appreciable reversion does not occur—bleach much more *quickly* than neutral solutions   This is true also of solutions buffered at pH 9 3 (Chase, 1935–36), so that it is a general alkaline effect and not one restricted specifically to ammonia

Pigmented Layers

The combined pigment epithelia and choroid layers of these marine fishes contain vitamin A, flavine, and an unidentified xanthophyll

Flavine was first found in fish pigment epithelia by von Euler and Adler (1934), nothing in the present investigation adds materially to the description of it given by these authors   The spectrum of flavine from bass pigmented tissue is shown in Fig 4

Fish xanthophyll is spectroscopically different from that found in frogs, and so will be described in some detail   It occurs in the tissues as an ester   when partitioned between 90 per cent methanol and benzine, it accumulates in the benzine layer before and in the methanol layer after saponification   It is readily extracted from strongly alkaline aqueous alcohol with benzine   The free pigment is strongly adsorbed on a column of calcium carbonate, forming a golden layer These are general properties of the hydroxycarotenoids or xanthophylls

In CS_2 the pigment possesses absorption maxima at 439, 470–472, and 500–501 mμ   A scup preparation diverged slightly from this, showing bands at 439, 476, and 502 mμ, but this spectrum was unusually diffuse as though the pigment had deteriorated in solution The spectrum of free xanthophyll from bass is shown in Fig 5

In general form these spectra resemble those of the dihydroxycarotenoids, $C_{40}H_{56}O_2$ The band positions, however, are closest to those of violaxanthin and taraxanthin, isomers of composition $C_{40}H_{56}O_4$ Violaxanthin yields a blue color when treated in ether with 25 per cent HCl   A single trial of this test with a sea robin extract was negative, possibly due to low concentration of the pigment
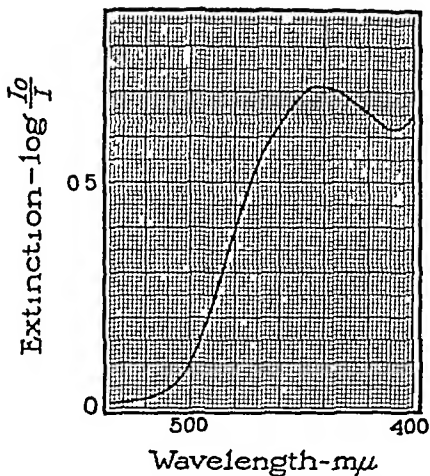
Fig 4 Spectrum of an aqueous solution of flavine from sea bass pigmented layers
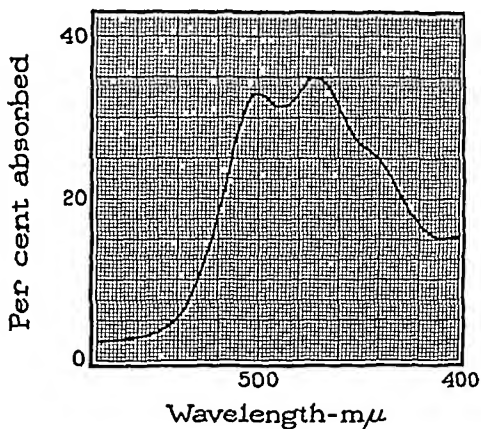
Fig 5 Spectrum of free xanthophyll in CS_2 from sea bass pigmented layers The non saponifiable portion of the crude extract had been partitioned between benzine and 90 per cent methanol The methanol fraction is shown

The distribution of the fish pigments between benzine and various concentrations of methanol is identical in all three species   No appreciable quantity of pigment leaves the benzine for 70 per cent methanol, a minor proportion does so for 80 per cent methanol, and practically all of it goes into 90 per cent methanol   This behavior is typical, and, indeed, roughly specific for the dihydroxycarotenoids

The relative solubilities of xanthophylls in polar solvents like methanol compared with non-polar solvents like benzine rise rapidly with the number of oxygen atoms in the molecule   This relation is shown in Table I, compiled from various portions of Zechmeister's monograph (1934), the behavior of the $C_{40}H_{56}$ carotenes is included   The terms epiphasic and hypophasic refer respectively to pigment solubility in the upper, benzine, and in the lower, alcoholic layers

TABLE I

Carotenoid	Partitioned between benzine and —	Behavior
$C_{40}H_{56}$	90 per cent methanol	Almost wholly epiphasic
$C_{40}H_{56}O$	95 per cent methanol	Partly hypophasic
$C_{40}H_{56}O_2$	90 per cent methanol	Almost wholly hypophasic
	70 per cent methanol	Almost wholly epiphasic
$C_{40}H_{56}O_3$	" " "	Slightly (1/9) hypophasic
$C_{40}H_{56}O_4$	" " "	More (1/6) hypophasic
$C_{40}H_{56}O_6$	" " "	Almost wholly hypophasic

The fish pigment therefore resembles the $C_{40}H_{56}O_2$ xanthophylls Its spectrum, however, is almost uniformly displaced 4–7 mμ below that of lutein, the most similar known xanthophyll of this composition   More specific identification of the fish pigment, only very small quantities of which were available in impure condition, is at present impossible

Cunningham and MacMunn (1893) and Lonnberg (1933–34) have found carotenoids in the skins, fins, and other tissues of a large number of species of marine fishes   The species investigated by Lonnberg form two groups, one in which the carotenoids resemble lutein spectroscopically, and an equally large group in which the spectra are displaced 4–6 mμ toward shorter wavelengths   It is not improbable that the second type of pigment is identical with that found in the present investigation

Sumner and Fox (1933) have found that the carotenoid pigments of superficial and deep structures in four species of marine fishes are exclusively of the xanthophyll series. It seems that, as in the frog, these pigments are generally distributed about the organism, and that their presence in the pigmented layers of the eye is probably of no special significance.

Rough estimates of the quantities of xanthophyll, vitamin A, and flavine in the pigmented tissues are presented in Table II. These were measured with a Pulfrich photometer in the manner already described (Wald, 1935–36a, b). In the case of xanthophyll, the same factor for converting photometer readings into absolute units was used as in the frog, since Kuhn and Brockmann (1932) have shown

TABLE II

Fish	No of eyes	Xanthophyll per eye	Vitamin A per eye	Flavine per eye
		γ	γ	γ
Sea robin	14	1 0	Trace	9
Scup	20	0 56	0 8	18
Sea bass	6	3 0	5	28

that a number of xanthophylls have approximately the same depth of color

SUMMARY

1 Visual purple from the sea robin, sea bass, and scup is almost identical spectroscopically with that from frogs. The interrelations of this pigment with vitamin A and retinene are also the same as in the frog

2 In strong acids or at pH > 11, the visual yellow of sea robin retinas is converted irreversibly into a pH indicator, yellow in acid and almost colorless in alkaline solution. Unlike neutral visual yellow, the indicator is not removed to form either vitamin A or visual purple. In the ammoniacal retina the reversion of visual yellow itself to purple is accelerated

3 The combined pigment epithelium and choroid layer in these fishes contain vitamin A, flavine, and an unidentified xanthophyll

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THE COLOR VISION OF DICHROMATS*

I Wavelength Discrimination, Brightness Distribution, and Color Mixture

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I

Purpose of Investigation As a Whole

Our interest in colorblindness springs from the desire to determine the unknown spectral sensibilities of the retinal cones, and to correlate with them the quantitative properties of *normal* color vision   This is a complex task *(cf* Hecht, 1930, 1931, 1932), because it involves the manipulation of nine variables   Of these only five or six have actually been measured, the others are hypothetical   Therefore the value of the theoretical treatment is always uncertain

Colorblindness is a simpler form of color vision because the color blind recognizes fewer colors than the normal, and his spectrum may be matched with two primaries instead of the three required for the normal (Young, 1807)   A complete description should therefore be possible in terms of four variables, obtained by measuring four independent aspects of colorblindness   Moreover, since Thomas Young it has been generally recognized that the various types of colorblindness[1] are most probably derivatives of normal color vision   Hence,

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[1] In order that our references to different types of colorblindness be easily understood we give here a diagnostic classification of all kinds of color vision, based on the accumulated knowledge of a hundred years and not on any theory

I MONOCHROMATS are persons who confuse any part of the spectrum with any other part, and who can match any part with white   There are (a) *Scotopic*

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information obtained from such a study should serve as a critical supplement to the theoretical treatment of the data from color-normals

With certain exceptions, the measurements necessary for our purpose have not been available   We therefore began the study of color-blindness to supply them   Our aim has been to investigate as many properties as possible with a few selected individuals   The results so far obtained are presented in the present group of papers

<div align="center">II</div>

Apparatus

All our measurements were made either with a Helmholtz Color Mixer (Koenig and Dieterici, 1892) built by Schmidt and Haensch, or with an apparatus composed of two monochrometers   A diagrammatic representation of the optical essentials of both arrangements is shown in Fig 1 where *A* describes the Helmholtz Color Mixer and *B* the two-monochrometer apparatus

The Helmholtz Color Mixer is essentially a spectrometer having one telescope and two collimators each with a light source   A 100 watt concentrated filament lamp *L* illuminates a finely ground glass *G*, placed 5 cm from it, which is then

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Monochromats, who have a brightness distribution in the spectrum corresponding to rod vision, and (*b*) *Photopic Monochromats,* who have a brightness distribution in the spectrum corresponding to cone vision

II DICHROMATS are persons who confuse large sections of the spectrum, who can match a particular part of it (either in the blue-green or in the yellow) with white, and who can match any part of it with a mixture of two primaries   Of these the (*a*) *Protanopes* confuse green, yellow, and red, they match a point in the blue-green with white, in particular, their brightness distribution in the spectrum even at high intensities is depressed in the red, and they are therefore frequently called red-blind   They are to be distinguished from the (*b*) *Deuteranopes,* who also confuse green, yellow, and red, and similarly have a point in the blue-green which they match with white, by the fact that the deuteranopes have a brightness distribution in the spectrum much like the normal   These are often but incorrectly called green-blind   To be distinguished from both of these green-yellow-red confusers are the (*c*) *Tritanopes,* who confuse blue and green, and match a point in the yellow with white   These are often called violet-blind or blue-blind, but such names involve a theory of colorblindness which is probably incorrect

III ANOMALOUS TRICHROMATS are persons who confuse parts of the spectrum, but who still require three primaries to match the spectrum   They form intermediates of all grades between dichromats and color-normals, and may be (*a*) *Protanomalous,* or (*b*) *Deuteranomalous,* or (*c*) *Tritanomalous,* depending on which type of dichromat they resemble

IV TRICHROMATS possess normal color vision

focussed by a condenser through a Nicol prism N of the Glan Thompson variety on to the slit S of the collimator   Between the slit and the collimator lens is a Rochon prism R whose position may be set anywhere in the collimator tube by a
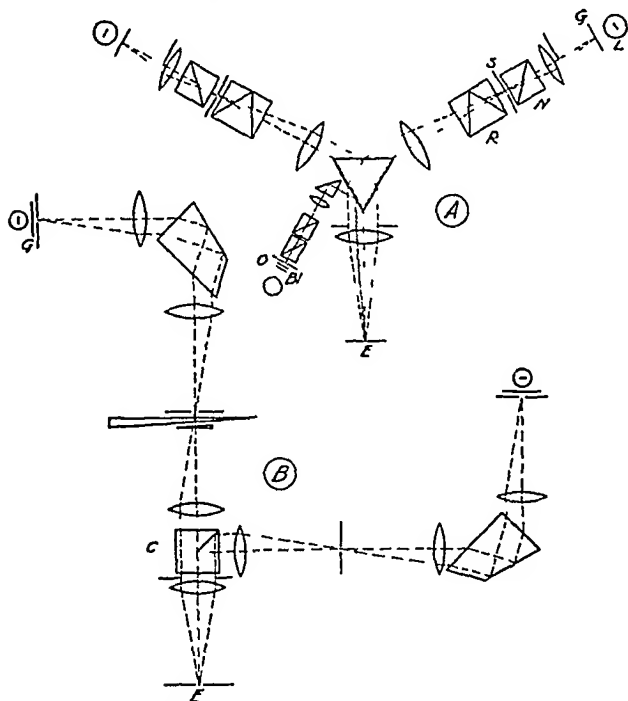
FIG 1 Diagrammatic representation of the optical arrangements A shows the main features of the Helmholtz Color Mixer, while B shows the apparatus composed of two separate monochrometers and a photometric cube

rack and pinion sliding over a scale   The eye at the exit pupil E of the telescope sees the two prism faces as contiguous semicircular fields, one from each collimator   The field as a whole has a diameter of 1 2°, and thus falls entirely in the rod free area of the fovea

The Rochon prism splits the beam of light into two components polarized at right angles   The ordinary beam passes through undeviated, the extraordinary beam is deviated at a constant angle   At the exit pupil of the telescope, the λ of the ordinary beam depends solely on the angular position of the collimator, while the λ of the extraordinary beam depends in addition on the position of the Rochon prism   The angular position of each collimator is set by a Brown and Sharpe micrometer screw which we added to the instrument, but which is not shown in the diagram   The light from the collimator slit is plane polarized by the Nicol prism, the angle of the prism then determines the fraction of each beam which passes to the exit pupil   In this way different amounts of light of two chosen wavelengths may be made to appear superimposed in each half of the visual field Their combined intensity may then be controlled by the collimator slit which is symmetrical and whose width may be accurately set by an appropriate microm-eter screw

When only one band of homogeneous light is needed from the collimator at the exit pupil, the Rochon prism is placed close to the collimating lens, thus com-pletely excluding the extraordinary beam at all positions of the collimator   The Rochon now acts like a Nicol prism and enables the ordinary beam to be varied in intensity by the Nicol prism, and the color mixer becomes an ordinary double spectrometer

We have added an arrangement for reflecting white light of color temperature 5000°K from the left prism face, its intensity is controlled by a pair of Nicol prisms The color temperature was achieved with a selected blue glass Bl, a piece of ground glass O, and a three-volt battery lamp whose amperage was adjusted by color-matching the combination against a lamp of standard color temperature plus a Davis-Gibson standard filter (Davis and Gibson, 1931)

The arrangement shown in B of Fig 1 furnishes to the eye at the exit pupil E a bipartite, circular field also 1 2° in diameter, each part being illuminated by homogeneous light from a separate, constant deviation spectrometer   The circular field is produced by the photometer cube C Each spectrometer is illumi-nated by a 100 watt lamp and ground glass G The intensity of one field is varied by a neutral gelatine wedge and balancer

Both pieces of apparatus were calibrated with sources of known wavelength In particular, the Helmholtz Color Mixer was calibrated frequently, because it showed a tendency to vary over long periods

<div align="center">III</div>

<div align="center">

Wavelength Discrimination

(A) Previous Work

</div>

The color-normal can divide the visible spectrum into about 180 short stretches which differ in appearance even when their brightness differences have been eliminated   The size (Δλ) of these spectral patches is not uniform, but shows two distinct minima found by all

observers,—in the blue green between 490 and 500 mμ, and in the yellow between 570 and 580 mμ, where $\Delta\lambda$ is about 1 mμ Nearly all observers show also one or two secondary minima in the violet at 440 mμ, and in the orange near 620 mμ, where $\Delta\lambda$ is between 2 and 3 mμ At the two ends of the spectrum $\Delta\lambda$ quickly rises to about 7 mμ (for a summary of the literature see Judd, 1932, Ladekarl, 1934, and Wright and Pitt, 1934)

Colorblinds possess only one minimum of $\Delta\lambda$ in the spectrum Brodhun (see Koenig, 1903 a) found this minimum near 500 mμ for the deuteranope Here $\Delta\lambda$ is about the same as for the color normal, but to either side $\Delta\lambda$ rapidly becomes large This has been confirmed by Steindler (1906), by us (Hecht and Shlaer, 1934), by Ladekarl (1934), and very recently by Pitt (1935) Steindler reported the same minimum near 500 mμ for deuteranopes and protanopes, and this has also been corroborated by later work (Laurens and Hamilton, 1923, Rosencrantz, 1926, Sachs, 1928, Hecht and Shlaer, 1934, Ladekarl, 1934, and Pitt, 1935) In addition, Steindler found a second minimum for protanopes at about 575 mμ, this, however, was undoubtedly due to lack of brightness control, because it has not been found by those investigators who controlled this source of error (Laurens and Hamilton, Sachs, Hecht and Shlaer, and Pitt) *Measurements with an extreme case of tritanomaly by Engelking (1925) show a minimum between 575 and 600 mμ, and indicate that a tritanope would probably have a minimum in that region of the spectrum*

With the exception of Steindler's measurements, which suffer because brightness differences were not eliminated, all of the published measurements are restricted to a small region of the spectrum covering about 30 mμ to either side of the neutral point near 500 mμ For a complete description, it is obviously necessary to have data which cover the whole spectrum We have measured two deuteranopes and one protanope[2] for this purpose

(B) Method

In making the measurements we set the wavelength of both halves of the field, while the subject determined whether by varying the brightness of one side only he could match the two sides perfectly   Light of λ_1 was first put on one side, then lights of other wavelengths were successively put on the other side until the wavelength λ_2 was found beyond which the observer could not match with λ_1 Each final observation was checked at least once before being recorded   The difference between λ_1 and λ_2 is $\Delta\lambda$ and represents the just discriminable interval

The subject was light-adapted throughout, and given a few minutes rest between each determination of $\Delta\lambda$ All judgments were made by looking freshly into the exit pupil, since differences which are apparent at once tend to disappear on prolonged examination   Measurements never lasted more than 2 hours and were interrupted by two or three 15 minute periods of relaxation

All the measurements were made with the Helmholtz Color Mixer, except the November series for the protanope which was made with the two-monochrometer system   The exit slit in both arrangements was kept at 0 4 mm   This is a compromise involving the desire to have a high purity of spectrum which demands a narrow slit, the elimination of diffraction which requires a wide slit, and a good brightness which also requires a wide slit   Since the homogeneity of the spectrum at the exit slit is maximal when collimator slit and exit slit have the same width, it is useless to have a fine collimator slit with a wide exit slit as used by Pitt (1935) We kept the collimator slits at 0 5 mm   With the Helmholtz Color Mixer, the exit pupil contained a band 4 mμ wide at 500 mμ, with the monochrometer system it was somewhat less   The brightness at the eye under these conditions is equivalent to between 200 and 500 millilamberts viewed through a 2 mm pupil, or between 2000 and 5000 photons

(C) Measurements

Table I gives the data for the two deuteranopes and the protanope The April data for A W G were secured at the beginning of his work After measuring a variety of visual properties, he made the May determinations   S R F made only one set of measurements   H J made two sets, the first at the very beginning, and the second several months later after having acquired skill, but after an absence of about 2 months from the laboratory   Each item in the table is the average of at least two separate measurements, in the region between 540 and 580 mμ the daily variation was such that we made four or five, and occasionally more determinations for one point

The table shows that for an individually variable stretch of the spectrum between 480 and 550 mμ, $\Delta\lambda$ for the colorblind is of the

same magnitude as for the normal To either side of this stretch $\Delta\lambda$ rises very rapidly, reaching nearly 50 mμ at the two ends The nature of the data is best illustrated in Fig 2, where $\Delta\lambda$ is plotted against both λ_1 and λ_2 The upper box of Fig 2 also shows for comparison $\Delta\lambda$ for the normal eye of Laurens (Laurens and Hamilton, 1923)

TABLE I

Wavelength Discrimination of Dichromats

Deuteranope A W G						Deuteranope S R F			Protanope H J					
April 1931			May 1931			June 1931			May 1933			November 1933		
λ_1	λ_2	$\Delta\lambda$	λ_1	λ_2	$\Delta\lambda$	λ_1	λ_2	$\Delta\lambda$	λ_1	λ_2	$\Delta\lambda$	λ_1	λ_2	$\Delta\lambda$
421 3	470 4	49 1	423 8	471 8	48 0	425 0	468 0	43 0	438 9	469 6	31 7	449 9	464 6	14 7
428 7	470 5	41 8	434 1	475 6	41 5	440 0	471 8	31 8	450 0	473 6	23 6	458 1	469 5	11 4
442 6	476 4	33 8	443 8	476 6	32 8	450 0	471 8	21 8	458 8	477 3	18 5	469 1	473 0	3 9
455 2	480 3	25 1	453 6	477 8	24 2	460 0	474 5	14 5	470 3	479 9	9 6	481 2	482 5	1 3
462 2	482 4	20 2	462 4	479 4	17 0	470 0	478 9	8 9	480 5	485 4	4 9	485 8	486 5	0 7
468 1	481 6	13 5	472 8	482 9	10 1	474 5	480 0	6 4	485 6	487 5	1 9	491 8	492 0	0 2
482 6	486 4	3 8	483 9	486 3	2 4	480 0	482 4	2 4	489 6	491 2	1 6	495 3	496 0	0 7
492 3	494 2	1 9	491 7	492 5	0 8	489 6	490 2	0 6	496 2	497 2	1 0	500 2	501 0	0 8
500 3	502 4	2 1	505 0	505 5	0 5	498 9	500 0	1 1	499 6	501 1	1 5	504 8	506 3	1 5
508 9	511 0	2 1	517 8	518 8	1 0	509 0	510 0	1 0	506 8	511 1	2 4	513 3	515 2	1 9
523 3	525 8	2 5	524 4	525 5	1 1	519 4	520 0	0 6	516 8	521 5	4 7	523 7	525 2	1 5
531 6	537 3	5 7	530 3	533 4	3 1	529 2	530 0	0 8	526 9	532 0	5 1	532 0	534 8	2 8
537 4	563 0	25 6	535 0	541 7	6 7	537 0	540 0	1 3	538 6	553 4	14 8	540 8	544 0	3 2
540 5	572 8	32 3	541 7	555 2	13 5	547 1	550 0	2 3	549 7	576 0	26 3	550 6	553 3	2 7
543 4	593 5	50 1	547 1	580 0	32 9	553 2	559 0	5 8	554 1	602 8	48 7	560 0	561 8	1 8
			550 2	608 3	58 1	563 3	569 0	5 7				568 0	571 7	3 7
						570 2	580 0	9 8				570 1	582 6	12 5
						571 0	595 0	24 0				573 0	593 0	20 0
						572 5	610 0	37 5				583 5	604 2	30 7
						575 1	620 0	44 9						

The neutral points (A W G at 495 0 mμ, S R F at 500 1 mμ, and H J at 491 5 mμ) are marked with a vertical line in Fig 2 The part of the spectrum which the observer matches with white of 5000° K is usually a band less than 1 mμ wide, its midpoint is given by the line

Note that $\Delta\lambda$ is minimal near but not exactly at the neutral point The colorblind thus distinguishes wavelength best near the region of the spectrum which to him resembles white To either side of this, $\Delta\lambda$ increases with the distance from the neutral point, on one side more
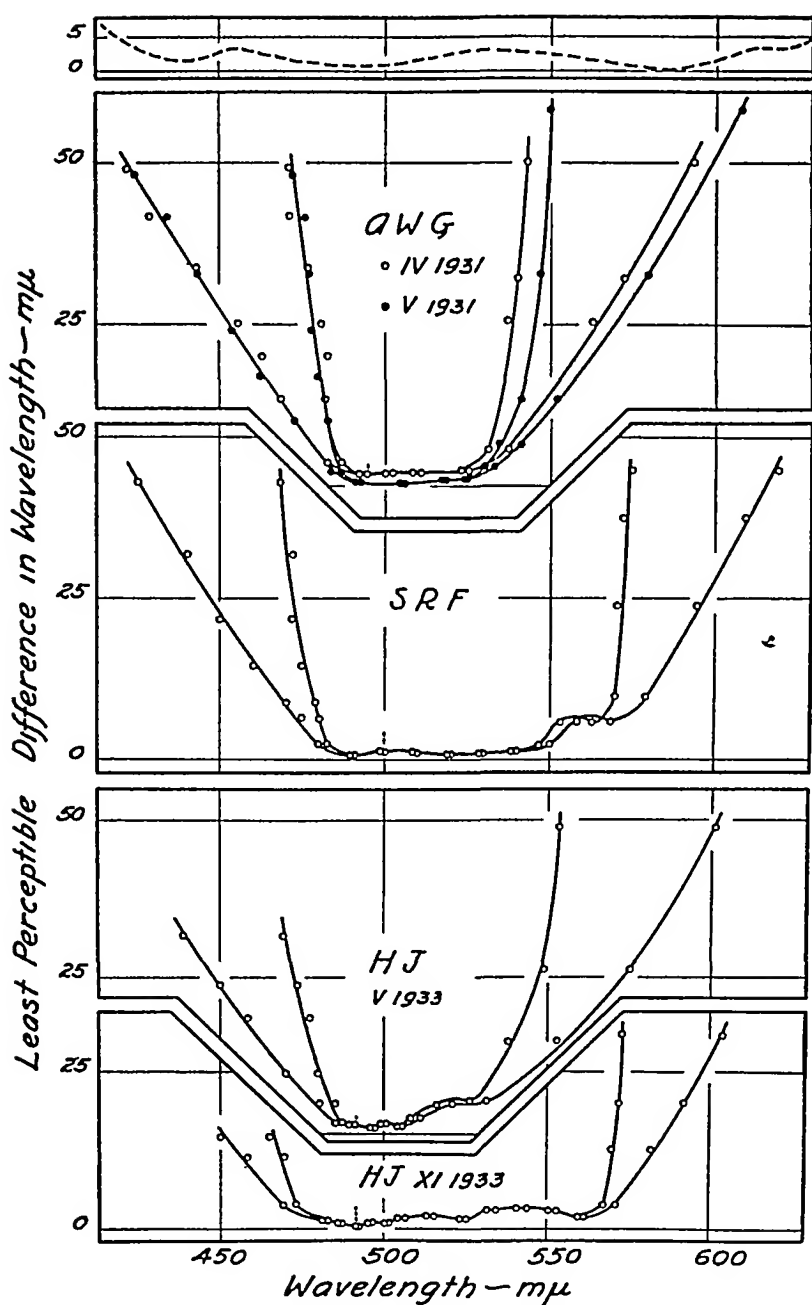
FIG 2 The data of wavelength discrimination for one normal (upper box) and for the two deuteranopes (A W G and S R F) and one protanope (H J) For the dichromats, Δλ is plotted against both λ₁ and λ₂

rapidly than on the other   The distribution of wavelength discrimination around the neutral point is not symmetrical, there is a more extensive stretch of moderately good discrimination toward the long wave end of the spectrum than toward the short end   The data of Laurens and Hamilton's protanope and of Sach's protanope, though quite meager, also show an asymmetrical distribution of Δλ around the neutral point, the asymmetry, however, is reversed in that the more extensive portion is on the blue side   Pitt's recent data, which cover only about a third the spectrum, indicate a more restricted region of good discrimination, distributed nearly but not quite symmetrically about the neutral point   The symmetry is probably due to Pitt's method which uses an average of Δλ to each side of a given λ   This is not a correct procedure, since Δλ is not the same in the two directions, as is obvious from Sach's work and from our data in Fig 2 Ladekarl's data resemble ours in the small range which he measured They are less symmetrical than Pitt's, but more than ours and Laurens and Hamilton's and Sachs'   Here again the method obscures the measurements because Ladekarl used the average error of setting method which automatically averages the two directions

The sharply rising inner lines in the data of Fig 2 represent two regions of striking sensory change   Starting with any wavelength below 460 mμ, our colorblinds see no differences in the spectrum until they hit this region of discrimination at about 470 mμ   Similarly, starting at the red end, they make no discrimination in λ until the region at approximately 570 mμ   The position of the sharply rising inner portion on the short wave side varies much less from time to time and from individual to individual than the one on the long wave side

From our measurements it appears that protanopes and deuteranopes show a very similar capacity for λ discrimination   It may be that when many individuals have been studied a consistent difference will appear over the whole spectrum similar to the very small and doubtful difference found by Pitt in the restricted region studied by him with six protanopes and six deuteranopes   However, the individual variation is so great that in terms of λ discrimination, either in a restricted region or over the whole spectrum, it is not possible to classify an individual as a protanope or a deuteranope

IV

Spectral Brightness Distribution

(*A*) *Normals and Dichromats*

Measurements of brightness distribution in the spectrum became of interest for colorblindness when it was found that the two classes of dichromat distinguished by Seebeck (1837) have a different brightness distribution in the spectrum   The deuteranope's brightness is much like the normal, whereas the protanope's is distinctly depressed in the red (Macé and Nicati, 1879, von Kries and Kuster, 1879, Donders, 1881)   Actually the protanope's brightness maximum is shifted toward the blue compared to the normal or deuteranope (Brodhun, 1887, Koenig, 1903 *b*, Abney, 1913, Exner, 1921, Kohlrausch, 1931, Pitt, 1935)

Since Langley's (1888) introduction of energy distribution data into spectral brightness determinations, the visual effectiveness of the spectrum for the color-normal has been repeatedly determined, and has become an established datum (Gibson and Tyndall, 1923)   Not so for the colorblind   Energy measurements in the spectrum are not easy to make, and investigators have been content to record the relative brightness distribution in a particular spectrum for the colorblind in comparison with the color-normal, sometimes (see especially Pitt, 1935) going to extraordinarily circuitous lengths to find the real shape of the data without making the energy measurements

We have determined the spectral brightness distribution for the three colorblinds recorded in the previous section, making our own energy measurements in the spectrum, and using a method which does not involve heterochromic photometry

(*B*) *Method*

Fig 2 shows that for the two large, end-stretches of the spectrum, the dichromat sees no differences in wavelength   Within these stretches the spectrum may therefore be compared in brightness without introducing any "color" differences [3]   But even in the region between 470 and 570, the just perceptible step Δλ, though

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[3] We use the term "color" here to include everything but brightness   Actually, as the following paper shows, the differences between contiguous wavelengths which the colorblind distinguishes are not concerned with hue, but with saturation

small, is finite and brightness comparisons can be made between points which differ by less than Δλ   This step by step method resembles the procedure of Gibson and Tyndall for the normal eye, but in addition it avoids all color differences for the colorblind

The Helmholtz Color Mixer was used for these measurements   We set a reference λ in the left collimator, and the subject determined by means of the Nicol prism the relative brightness of the same λ in the right collimator   All further relative brightness measurements were then referred to this λ in the right collimator as a standard   Keeping the reference λ in the left collimator constant, the brightness of a series of wavelengths in the right collimator was then measured by the subject, until a complete match between the two sides became impossible   The reference λ in the left collimator was then changed to a new reference λ, chosen so as to match the last λ measured in the right collimator   the brightness of the new reference was then determined relative to the last measured λ   A new section of the spectrum in the right collimator was then measured against the new reference λ in the left until a complete match on both sides again became impossible   A new reference, chosen as before, was then introduced into the left collimator   its brightness was calibrated as before, and a new section of the spectrum measured against it   The procedure was repeated as often as necessary to cover the spectrum   For those regions where Δλ is large, one reference λ easily served for 75 or 100 mμ, and determinations were made every 10 mμ   But between 470 and 570 mμ the reference λ had to be changed with increasing frequency until near 500 mμ it was changed for every other measurement   Usually three, and frequently five readings were made for each determination of relative brightness, for calibrating each new reference λ five and often ten readings were taken   About fifty steps were required to traverse the spectrum

We calibrated the energy distribution of the spectrum with a Hilger linear thermopile placed at the exit pupil of the telescope, and thus avoided corrections for the transmission of the prism and lenses, and for the dispersion of the spectrum   With the low resistance thermopile we used a Paschen galvanometer supplied by the Cambridge Instrument Company   The deflection of the galvanometer was carefully calibrated with known voltages   To increase the energy at the exit pupil both collimators were used at the same time, and the telescope lens was kept at full aperture   The slits were opened to 0 5 mm   Six readings were made at each point at 10 mμ intervals along the spectrum   The results secured were smooth so that a calibration curve could easily be drawn through them   The values necessary for correcting the measured, relative brightnesses were taken from the curve

(C) *Visibility Curves*

Table II gives the data, which are also shown graphically in Fig 3 It is apparent that with minor exceptions they are regular and smooth

TABLE II

Brightness Distribution in Spectrum   The Maximum in Each Case is Placed at 100

Deuteranopes				Protanope	
A W G		S R F		H J	
λ in mμ	Brightness	λ in mμ	Brightness	λ in mμ	Brightness
422 8	4 72	404 2	0 11	404 3	0 39
433 4	5 94	414 6	0 26	414 5	1 24
442 9	5 91	425 3	0 35	425 3	2 76
453 0	7 52	435 0	0 46	435 2	4 26
463 3	10 82	445 3	0 58	445 4	5 34
470 4	17 33	454 7	0 75	454 6	7 54
476 4	20 72	464 8	1 22	464 7	10 26
479 4	23 56	476 1	2 15	480 0	17 04
482 6	27 29	472 2	1 61	485 0	21 76
484 3	24 85	484 1	2 65	487 4	25 84
485 8	31 02	488 2	3 12	489 8	29 52
487 5	38 28	491 7	4 29	492 4	32 00
489 2	39 93	495 2	5 79	494 0	32 96
490 8	42 14	497 8	7 76	495 5	34 48
494 4	48 44	499 7	7 66	497 6	37 76
497 1	60 19	501 0	7 47	499 6	41 92
498 7	66 63	502 6	8 64	501 2	43 68
501 7	62 30	504 0	10 71	503 3	49 04
505 4	60 19	505 2	12 27	506 3	57 12
509 1	62 80	507 2	13 86	510 2	64 48
514 0	68 94	509 1	17 41	514 2	71 44
519 1	82 27	512 1	20 61	518 2	80 00
527 6	84 05	514 1	23 96	523 7	88 40
536 5	84 74	516 0	29 44	536 4	99 36
546 1	97 48	518 2	34 97	546 2	99 20
556 6	99 00	520 4	39 80	557 9	92 48
567 9	100 25	523 7	47 83	568 1	85 84
579 9	99 55	531 8	66 48	578 6	66 64
589 6	81 05	536 4	83 45	588 5	51 92
599 7	73 49	546 1	84 62	600 5	34 16
608 5	65 54	558 0	93 08	609 6	24 16
619 6	47 19	568 1	89 63	618 6	16 64
629 3	35 61	578 7	100 00	630 3	8 32
639 5	25 77	588 7	96 03	640 4	4 74
650 4	16 50	600 6	78 57	649 4	2 88
662 1	8 88	609 6	81 76	660 5	1 36
674 5	4 69	618 6	53 07	670 0	0 72
690 4	1 91	630 3	36 23	680 5	0 40
701 7	0 92	640 4	21 68	691 5	0 24
		649 4	16 69		
		660 7	8 64		
		670 4	4 80		
		680 6	2 81		
		691 3	1 33		
		700 0	0 80		
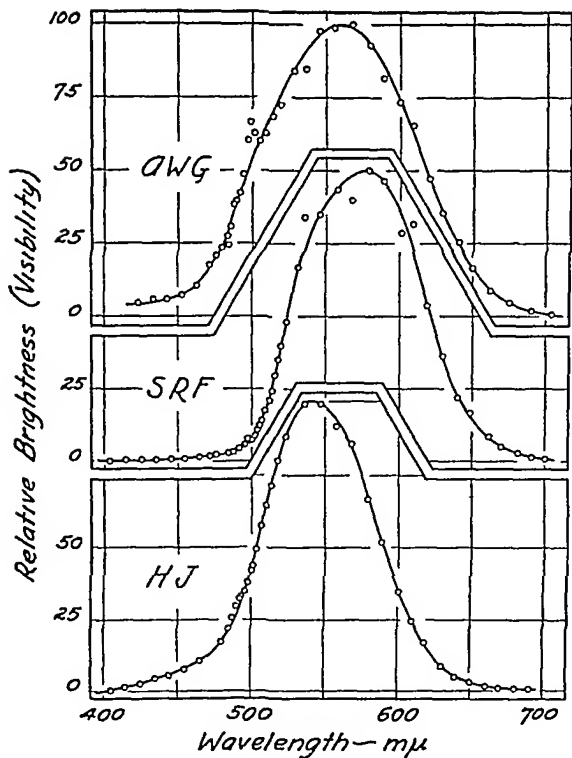
Fig 3 Brightness distribution in the spectrum for two deuteranopes (A W G and S R F) and one protanope (H J) The maximum for each has been arbitrarily placed at 100

Perhaps the best way of examining the visibility curves is to view them against the normal background In Fig 4 the stippled area represents the range of measurements for the 52 color normals investi

gated by Gibson and Tyndall The protanope H J barely falls within the normal range on the blue side of his maximum, and is definitely outside the normal range on the red side On the other hand, the deuteranope S R F barely falls within the normal range on the red side and is distinctly outside on the blue side The deuteranope A W G has a rather wide visibility curve which falls within the normal range on both sides Pitt's recent averages of six deuteranopes and six protanopes are included in Fig 4 H J is an almost
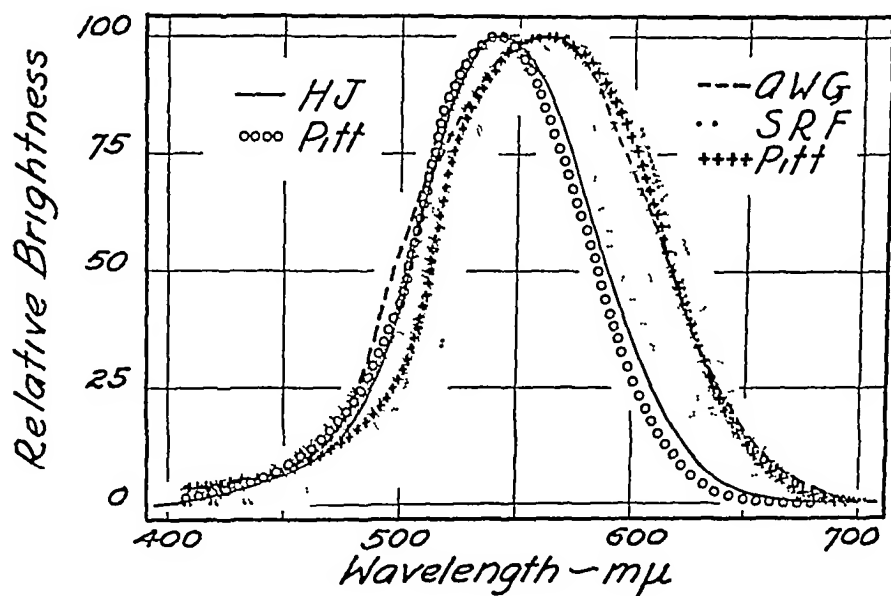
FIG 4 Brightness distribution in the spectrum The stippled background represents the range for fifty-two color-normals measured by Gibson and Tyndall (1923) A W G and S R F are our deuteranopes, and the crosses give the average of Pitt's (1935) deuteranopes H J is our protanope, and the circles give the average of Pitt's protanopes

perfect replica of the averaged protanope, his curve and the average curve are narrower than the normal and deuteranope curves A W G and S R F coincide with the averaged deuteranope on the red side, but lie to either side of the average on the blue side

The data show clearly that just as the protanope's curve is shifted toward the blue compared to the normal, so the deuteranope's curve is shifted toward the red, but not so much This has already been noted by Pitt

The least variable part of the visibility curves seems to be the red side of the maximum, and this furnishes the only reliable means of telling when a given individual is a protanope or a deuteranope (Donders, 1884, von Kries, 1897) We have adopted this as a diagnostic routine test, making a brightness comparison between 550 and 650 mμ The comparison is made in two steps, first measuring 550 against 600, and then 600 against 650, and thus avoids a heterochromic match The ratio of 550/650 mμ is large, near 35 for the protanope, and small, near 4 for the deuteranope Of the ten protanopes and twelve deuteranopes whose diagnostic brightness ratio we have measured, we have found no case falling far from these values

\
Neutral Point

The neutral point of a dichromat is that point in the spectrum which he can match with white light Using white of 5000° K we have determined the position of the neutral point for the twenty two dichromats just referred to The measurements were made exactly as with wavelength discrimination Half the field of the Color Mixer contained white, while the other had a succession of wavelengths whose brightness the subject could control He was required to state whether he could match the two halves or not The band in the spectrum which the dichromat can match with white is usually about 1 mμ wide

The measurements are shown in Fig 5, which includes not only our own data, but those of Koenig (1884) and of Pitt The figure shows that the position of the neutral point for the deuteranope is more widely scattered than for the protanope The extreme position of one neutral point at 525 mμ is authentic, we naturally questioned it, and repeated the determination

The average position of the neutral point for the twenty-one protanopes in Fig 5 is 496 5 mμ, for the twenty five deuteranopes it is 504 3 mμ The averages for our own cases are protanopes 498 2 mμ, and deuteranopes 510 2 mμ In spite of this distinct difference between the averages of the two types, the individual variation is so large that the neutral point of any single person cannot be used to

identify the type of dichromat he is   This is possible only in terms of his brightness distribution in the spectrum

With our protanope H J we have carefully investigated the effect of brightness on the position of the neutral point   In the brightness range between 25 and 5000 photons we could find no change in its position   Obviously our lowest intensity was well above that for which Koenig (1884) had found a gradual shift in the neutral point
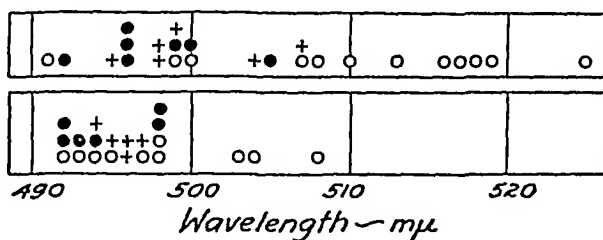
FIG 5 Distribution of neutral points for twenty-one protanopes (lower box) and twenty-five deuteranopes (upper box)   The open circles are our own measurements, the crosses are from Pitt (1935) while the solid circles are from Koenig (1884)

VI

Color Mixture

(A) Uniqueness of Mixtures

The most revealing characteristic of the dichromat is his capacity to match the spectrum with mixtures of only two primaries   The common formulation of this fact has been that just as a color-normal can match the spectrum with unique mixtures of three primaries, so a dichromat can match the spectrum with unique mixtures of two primaries   Our experience, however, has shown that the dichromat cannot give *unique* values in gauging the spectrum with two primaries

The reason for this lies in the data of wavelength discrimination as already shown in Fig 2   For the dichromat between 460 and 520 mμ, $\Delta\lambda$ varies from 1 to 6 mμ, but for the rest of the spectrum it becomes rapidly larger   For the spectrum below 450 and above 550 mμ the interval $\Delta\lambda$ varies from 10 to 50 mμ Thus, for example, since the dichromat cannot distinguish between 420 and 450 mμ, a mixture of two primaries made to match 450 mμ will also match 420 mμ provided

brightness differences are eliminated   The match for 450 mμ therefore cannot be unique   This is obvious for spectral regions where $\Delta\lambda$ is large, but it is equally true where $\Delta\lambda$ is small [4]   The dichromat cannot usually discriminate 510 from 515 mμ Hence a mixture of two primaries which matches 510 also matches 515 and cannot be considered unique for either

These considerations were forced upon us when we set out to gauge the spectrum of our dichromats with two primaries by the usual procedure which permits the subject to vary the combined brightness as well as the proportions of the two primaries   The results secured in this way were frequently indeterminate, depending on the brightness level, and forced us to adopt a wholly different procedure   We set a specified mixture of two primaries, and the subject determined the limits of λ which he could match with it by varying only the brightness of the mixture

(B) Procedure

The primaries were 458 7 and 570 0 mμ and were located in the right collimator of the Helmholtz Color Mixer, the latter by the position of the collimator as a whole and the former by the position of the Rochon prism in it   The Nicol prism in the right collimator determined the value of the mixture which appeared in half of the field   In the other half, one wavelength after another was tested to define the boundaries λ_1 and λ_2 between which the dichromat could match the mixture of primaries merely by controlling their combined brightness

Essentially this is the technic of λ discrimination, except that the standard in half the field is a mixture of two primaries   Moreover the range of λ_1 and λ_2 obviously includes two steps in λ discrimination because we measured the extreme matching positions to the short wave end and to the long wave end for each mixture   The width, however, is not twice that of a single step since the steps to either side are rarely equal

In this way we tested a series of mixtures, sufficient to cover the spectrum We maintained a roughly uniform brightness of between 2000 and 5000 photons throughout the spectrum by controlling the slit width and the Nicol prism of the homogeneous light in the left collimator   The collimator slit was no greater than 0 5 mm , the exit slit at the telescope was 0 4 mm   The energy content of the two primaries was determined as before with the Hilger thermopile and Paschen galvanometer, their relative brightness was then computed from the respective visibility curves in Fig 3

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[4] It deserves to be pointed out that the uniqueness of trichromatic matches for color normals is subject to the same limitations in spectral regions where $\Delta\lambda$ is larger than 2 or 3 mμ

(C) Mixture Data

Table III gives the data for the deuteranope A W G and for the protanope H J   We measured A W G twice, 1 month apart   The two series are so similar that it would serve no useful purpose to print

TABLE III

Color Mixture of Deuteranope A W G and of Protanope H J   Spectral Limits λ_1 and λ_2 Matched by Mixtures of Primaries 458 7 and 570 0 mμ

Energy ratio of primaries 458 7 mμ/570 0 mμ	A W G		H J	
	λ_1	λ_2	λ_1	λ_2
91 5	415 2	473 8	436 6	469 1
			439 1	478 5
67 1	422 9	472 0		
51 4	436 1	475 4	431 5	469 2
			438 2	470 4
32 8	447 9	475 4	440 2	468 0
22 8	466 1	477 4	446 6	469 1
	464 8	478 6		
12 7	474 6	482 2	451 9	474 2
8 08	481 6	485 4	454 4	474 8
3 99	489 9	492 5		
3 50			473 0	478 6
1 90	496 8	498 7	479 5	482 2
0 754	504 6	506 9	489 5	490 4
0 357	512 6	514 2	496 1	497 4
			495 1	496 8
0 243			502 0	504 1
0 177	519 1	520 3		
0 0837	525 2	528 8	509 3	511 4
0 0546	528 9	532 4	513 5	517 0
0 0333	531 4	541 0	518 5	522 7
0 0181	538 7	568 8	524 0	528 4
0 0114	537 1	606 1	527 5	532 8
0 00782	542 5	615 5	533 2	536 8
0 00497			533 2	542 4
0 00378			534 0	566 4
0 00277			535 6	581 3

them both   The data here given are from the second series   The table records the energy ratio of the two primaries and the limits between which the dichromat matches each of the mixtures   The relative brightnesses of the two primaries 458 7 and 570 0 mμ are 8 6

and 79 5 for A W G , and 9 4 and 99 0 for H J as taken from their visibility curves in Fig 3   Therefore to convert energy ratios into brightness ratios in Table III, A W G 's ratios are to be multiplied by 0 0950, and H J 's by 0 108,—in both cases by very nearly 1/10

The data are plotted in Fig 6, with the logarithm of the energy ratio as ordinates, and the limits λ_1 and λ_2 as abscissas   Because of the log plot, the shape of the relationship remains the same whether the ratio of the primaries is in terms of energy, or of brightness, or of arbitrary units   For rough conversion into brightness ratios subtract 1 from the log values of the ordinates

The data for the two subjects are not very different   In both there is a stretch between about 480 and 530 mμ where the mixture changes quite sharply with λ, and where the wavelength band corresponding to a specific mixture is very small   This is in keeping with the small values of Δλ found here in measurements of λ discrimination   To either side of this central stretch the matching band widens very rapidly, and this also is in harmony with the data of λ discrimination [5] The region of rapidly changing and sharply defined mixtures extends over about 2 log units of ratio of the primaries

For both subjects the center of this sharp region is about the same distance to the right of the neutral point, the center is at 507 mμ for A W G , and at 503 mμ for H J   For the deuteranope A W G the energy ratio corresponding to this point is 0 661, while for the pro tanope H J it is 0 191   In other words, for the protanope H J much less (in energy) of the 458 7 primary and much more of 570 0 primary are required than for the deuteranope A W G to match the region of sharpest discrimination   This difference persists when relative energy

[5] Near the two extremes of the mixture data where the interval Δλ is large, there are frequently found small patches of the spectrum perhaps 3 mμ wide, which the dichromat cannot match with the specific mixture of primaries used to match the spectrum to the right and left of the non matching patch   The position of these non matching islands is quite certain at any time, but is very variable from day to day   The data for these regions as given therefore represent the edges of the matching bands as found by starting from those wavelengths which the dichromat could not match, and working closer and closer until wavelengths were found which he could match   These non matching islands are not due to the apparatus or method because we did not eliminate them even after many variations in technic and apparatus
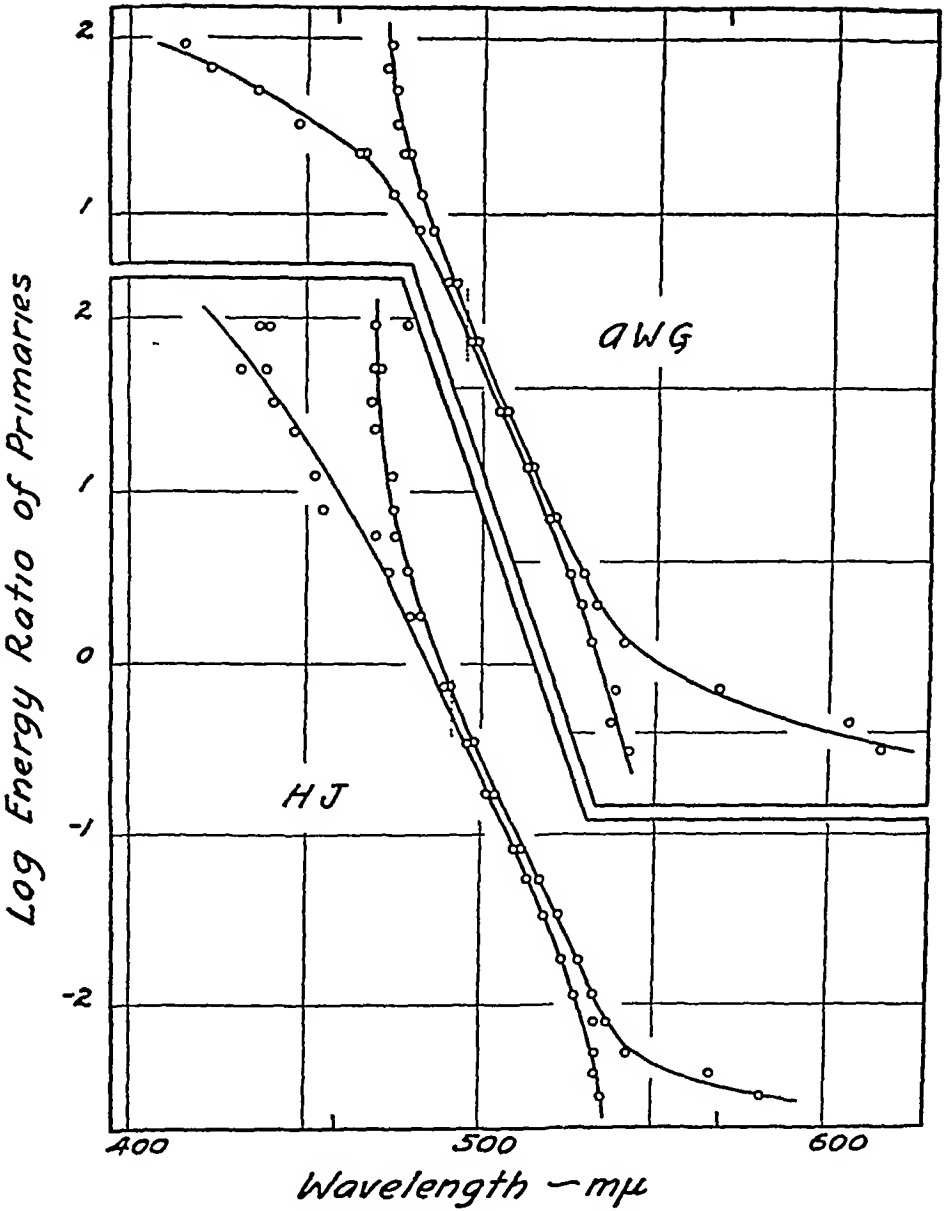
FIG 6 Color Mixture   The ordinates give the ratio of the amounts of the two primaries 458 7 and 570 0 mμ (shown on the abscissas) which are matched by regions of the spectrum included between the points   A W G is the deuteranope, H J the protanope

is replaced by relative brightness, because the conversion factors are very nearly the same in the two cases, and may indicate a specific difference between the two types of dichromat This difference is not to be confused with the minor difference in mixture ratios which Wright (1929) first failed to find and Pitt (1935) in his laboratory later did find between the two types of dichromat, Pitt's difference probably depends on the method of measurement which assumes the uniqueness already referred to

(D) Uniqueness and Brightness

Examination of Fig 6 shows that in the central stretch between 480 and 530 mμ the matching range is small, and a reasonable uniqueness may be claimed for a certain mixture matching a given λ Beyond this region no approach to uniqueness is possible, and the two edges of the matching band become more and more separated For example, 475 mμ is near the best λ discrimination of H J Yet as Fig 6 shows, the upper and lower limits for the matching mixture differ by over 0 3 log unit, that is by 100 per cent So rapid is the change here, that for 470 mμ the upper and lower limits already differ by over 0 75 log unit, that is by 600 per cent

This whole point has previously been overlooked and has resulted in the growth of certain false notions For example, both Koenig (1887) and Brodhun (1893) were led astray by it and concluded that Newton's law for the addition of brightness was not valid for dichromats They found, as had van der Weyde (1882) that for dichromats under certain conditions, color matches (mixtures vs homogeneous light) did not remain valid at all intensities Thus, if true, is surely a startling situation that must be interpreted and understood

Brodhun's work will illustrate the situation With the two primaries 460 and 615 mμ Brodhun, who was a deuteranope, gauged parts of the spectrum at different brightnesses For 480 and 490 mμ he found that the ratios of the primaries remained constant regardless of the illumination, while for 540 and 560 mμ the ratios varied strikingly with the illumination

We have repeated these experiments precisely as Brodhun made them, and there is no question of their truth The only difficulty with them is that they are meaningless They depend for their exist-

ence first on the non-uniqueness of color matches for dichromats
except for very restricted regions of the spectrum, and second on the
purely irrelevant fact that in most spectra the two primaries differ
considerably in brightness

The method nearly always employed is to place homogeneous light
in one side of a field, and two spectral primaries in the other side, and
then to ask the subject to vary the relative amounts of the two prima-
ries as well as their combined brightness in order completely to match
the homogeneous light   The dichromat thus has two problems
First, he must select a combination of the two primaries which will
resemble the homogeneous light, and second, he must change their
combined brightness to match it in brightness as well   The subject
does first the one, and then the other, repeating the procedure until a
match is secured

It is apparent from Fig 6 that at 500 mμ the vertically recorded
mixture range is very limited and, therefore, the ratio setting will
be almost unique because a small change in the relative amounts of
the two primaries will change the recognizable appearance of the
mixture   At 540 mμ, however, the mixture range which will produce
a match is tremendous   Since the relative brightness of the two
primaries is very different, varying the ratio of the primaries also
varies the total brightness, and a match can be achieved by this means
alone   At low intensities, the match will be made mainly by reducing
the brightness of the brighter long-wave primary, and the resulting
ratio of long to short-wave primaries will be small   At high intensities
the match will be made mainly by increasing the brighter primary,
and the ratio of long to short-wave primaries will be large   This is
what Brodhun found, but it is due basically to the great range of mix-
tures which can match 540 mμ, and not to the failure of the third law
of color mixture

We have repeated Brodhun's measurements with our procedure,
and the results are as expected   His primaries (416 and 615 mμ)
were in the right collimator, and 560 mμ in the left   With the right
Nicol prism we set a specific ratio of the two primaries which we knew
easily matched 560 mμ for the dichromat, and this ratio remained un-
disturbed throughout the experiment   The brightness of 560 mμ
was then set at a specified value by means of the left Nicol prism

The dichromat (A W G) was then asked to make the two fields match merely by adjusting the slit of the right collimator which controls the combined brightness of the two primaries Three readings were made The brightness of 560 mμ was then increased by changing the left Nicol prism, and A W G again matched it by increasing the common slit of the two primaries In this way we varied
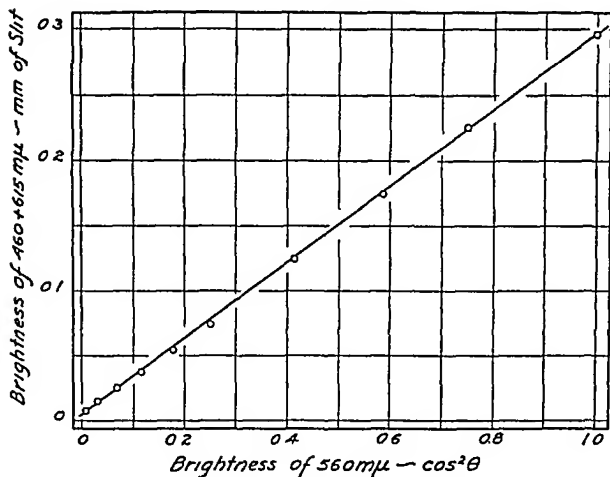
FIG 7 Brightness and color mixture There is a linear relation between the combined brightness of the mixture of the two primaries and the brightness of the homogeneous light of 560 mμ which matches them

the brightness of 560 mμ in ten steps over a range of 1 to 14, the dichromat making three separate readings at each intensity

The data are in Fig 7 The abscissas give the intensity of λ560 as $\cos^2 \theta$ of the Nicol angle, while the ordinates give the combined intensity of the primaries in terms of slit widths in millimeters It is obvious that the two are related linearly, that as the brightness of 560 mμ is increased it is necessary to make a corresponding increase

in the brightness of the primaries without changing their relative
proportions   We made this type of experiment three times using
different primaries and different homogeneous lights, and the results
all showed the same thing   In one experiment we even increased the
intensity range by a factor of 10 without finding any different result
Therefore, a given mixture will match a given homogeneous light
regardless of brightness [6]

SUMMARY

1   Protanopes and deuteranopes show one maximum of wavelength
discrimination which occurs near their neutral point in the region
of 500 mμ (blue-green for color-normal)   The value of the just dis-
criminable wavelength interval $\Delta\lambda$ is about 1 mμ at this point and is
much like the normal   To either side of this, $\Delta\lambda$ rises   It increases
rapidly on the short-wave side, and slowly on the long-wave side,
rising to about 50 mμ at the two ends of the spectrum

2   The brightness distribution in the spectrum for dichromats falls
only partly outside the range established for color-normals   The
protanope curve is narrower than normal, and its maximum lies nearly
15 mμ to the left of it   The deuteranope curves are about the same
width as the normal, and their maxima lie slightly but definitely to
the right of it   The main difference between protanope and deu-
teranope spectrum sensitivity lies on the red side of brightness curves,
where the deuteranope is strikingly higher   This difference furnishes
the only reliable diagnostic sign which may be applied to an indi-
vidual dichromat for separating the two types

3   The average position for the neutral point of twenty-one pro-
tanopes is 496 5 mμ, of twenty-five deuteranopes 504 3 mμ The
range of variation in the position of neutral point is twice as great for
the deuteranope as for the protanope

4   Dichromatic gauging of the spectrum cannot yield unique mix-
ture values for any wavelength because of the large stretches of poor
wavelength discrimination   Data have therefore been secured which
locate the spectral ranges that can match specific mixtures of two
primaries when brightness differences are eliminated   The form of

[6] Obviously this is true only for strictly foveal fields where the Purkinje phe-
nomenon is avoided, as has been done in all the measurements recorded here

the data is much the same for a protanope and for a deuteranope, the only difference is in the relative brightness of the primaries.

5 Previously accepted anomalies in the spectral matching of di chromats which have led to the rejection of the third law of color mixture for them, have been eliminated   They are shown to have been due to the non uniqueness of color matches and the usually disparate brightnesses of the primaries   Color mixture matches for dichromats are valid at all brightnesses

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THE COLOR VISION OF DICHROMATS

II Saturation as the Basis for Wavelength Discrimination and Color Mixture

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I

Wavelength Discrimination as Saturation

The three variables of color vision are hue, saturation, and bright
ness The characteristic color differences along the spectrum which
the color normal sees are essentially concerned with hue In measure
ments of wavelength discrimination the precise value of the just per
ceptible difference in wavelength at constant brightness is no doubt
influenced by saturation differences, but the major factor is hue
What determines wavelength discrimination in the colorblind? Since
the best λ discrimination of protanopes and deuteranopes occurs near
500 mμ, which is a region matched with white, saturation may be a
more important factor for them than for the color normal

A simple but striking experiment demonstrated to us that saturation
is indeed the determining factor [1] One side of the field in the Helm

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[1] Actually we arrived at this notion entirely from theoretical considerations
Adopting the quantitatively formulated trireceptor idea of Young (1807) as a
basis for color vision (Hecht, 1930 1934) and supposing that in colorblindness one
of the cone primaries, say the red is transformed into one of the other two, say
the green then three things follow First near 500 mμ all three curves will inter
sect in one point, the resulting sensation will be white and will correspond to the
neutral point Second to the left of this neutral point, where the blue primary is
higher than the other two the sensation will be (a) white by virtue of those effects
where the blue green, and red primaries have the same height, and (b) blue by
virtue of the excess effect of the blue primary Different portions of the spectrum
between the neutral point and the short wave end will then differ only in the rela
tive amounts of blue and white that is, in saturation Third, on the right side
of the neutral point, the now similar red and green primaries are higher than the

holtz Color Mixer is set for 520 mμ (green to us) while the other side
is set for 650 mμ (red)   The colorblind cannot match these two fields
merely by adjusting their brightness differences   However, when a
little white light is added to 650 mμ, the colorblind at once reports the
two fields to be very nearly matched, and further slight additions of
white remove all differences between the two fields to the colorblind,
though to us they are almost as widely different as before   The same
experiment may be made with 480 (blue-green) and 420 mμ (violet),
but it is not so striking because these two wavelengths are not so
sharply different to us as red and green

Though we made this discovery quite independently on the basis of
theoretical argument, examination of the literature showed that the
phenomenon had already been found by von Kries and Kuster (1879)
However, no quantitative investigation has ever been made of it
We therefore measured the situation throughout the spectrum with
our protanope H J in the hope of supplying a new type of data for
the color vision of the colorblind

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II

Procedure

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For matching the short-wave side of the spectrum, we used mixtures of 440 mμ
with white of 5000°K   For the long-wave side, we used 650 mμ with the same
white, for which on some occasions we substituted the white of the neutral point
at 491 6 mμ

The procedure was essentially the same as with measurements of λ discrimina-
tion described in the preceding paper   A mixture of white and 440 mμ or of
white and 650 mμ was placed in half the field of the Helmholtz Color Mixer   In
the other half, light of a given wavelength was placed, and the subject reported
whether he could match the two fields merely by varying the brightness of this
light alone   Successive wavelengths were tried until the values of λ_1 and λ_2 were
located between which the subject could match the mixture of 440 mμ (or of 650
mμ) and white in the other half of the field   We found it convenient to locate the
two edges of this matching band by working from non-matching regions on each
side to the matching region between them   The readings were clearer than by

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blue   Everything below the blue curve represents white, everything between
the blue curve and the identical red and green curves is yellow   The different
wavelengths then produce merely different relative effects of yellow and white,
again this means differences in saturation only

working steadily across the matching region until reaching the non matching portion this has already been referred to in the preceding paper

A series of known mixtures were measured in this way covering the spectrum We always set the mixture and the wavelength of the spectral light, while the subject controlled the brightness of the spectral light and made the judgment of match or no match

In order to record the data quantitatively it was necessary to determine the relative brightnesses of the 5000°K white, of the neutral point white, of 440 mμ, and of 650 mμ These were measured by H J himself The 5000 K white light was put into the left half of the field, and homogeneous light of the neutral point (491 6 mμ) into the other, and their relative brightness measured The whole white light was then removed and 491 6 mμ from the right collimator placed in its stead and its brightness measured in terms of the 491 6 in the other field From this we found the relative brightness of the white and the 491 6 on the same side of the field The relative brightnesses of 491 6, 440, and 650 mμ as they occur in the Color Mixer were taken from H J's measurements of brightness distribution in the spectrum already recorded in the preceding paper Knowing these values we were able to make mixtures corresponding to any desired brightness ratio

III

Saturation Distribution

The data are in Table I, and record the two positions in the spectrum, λ_1 and λ_2, between which H J was able to match the specific mixtures of white and 440 mμ or of white and 650 mμ given in terms of the brightness ratio of color to white Those mixtures which were made with 650 mμ plus the neutral point (491 6 mμ) instead of whole white light, are printed in italics The four measurements in parentheses were made by the method of working through the matching band rather than from both sides of the band

The best way to grasp the meaning of the data is to examine Fig 1 in which λ_1 and λ_2 are plotted against the logarithm of the brightness ratio of the mixtures, and to compare this figure with Figs 2 and 6 of the preceding paper There is at once evident a close correlation between these data and those of λ discrimination and of color mixture The horizontal distance between the pairs of lines to the left and right of the neutral point is really a measure of Δλ (including two discrimina tion steps), because a mixture which matches a given λ will naturally also match those values of λ which fall within the band Δλ previously found In Fig 1 the Δλ band is narrow near the neutral point, to the

TABLE I

Limits (λ_1 and λ_2) of Spectral Bands Matched by Protanope H J with Mixtures of White Light Plus Either 440 mμ or 650 mμ

440 mμ and white			650 mμ and white		
Brightness ratio 440/white	λ_1	λ_2	Brightness ratio 650/white	λ_1	λ_2
70 S		473 3	166 0	572 9	
36 3		472 5	129 0	573 0	637 7
17 S		475 2	110 0	569 0	
9 12		473 2	79 4	560 1	
5 50		474 0	55 0	545 2	
3 16		473 6	43 7	563 2	629 0
1 59		474 0	38 9	538 6	
1 59		474 0	38 0	525 3	
0 759		475 7	27 5	521 0	
0 741	424 6	(467 4)	26 9	524 4	
0 3S0		476 1	26 9	526 0	
0 372	443 3	(466 4)	20 9	523 1	
0 324		478 0	20 4	517 3	554 0
0 246	459 0	(470 7)	15 5	515 0	
0 246		478 8	13 5	515 4	
0 246		4S0 4	10 7	512 0	524 9
0 135	464 6	(474 7)	9 33	511 2	
0 135		478 0	8 91	510 7	513 8
0 05S9	473 1	4S3 0	5 50	507 3	
0 05S9		4S0 4	4 37	506 0	509 2
0 05S9		4S3 3	4 37	504 2	506 3
0 0219	4S2 S	4S6 0	3 16	502 7	503 6
0 0219		4S6 0	2 95	501 3	503 3
0 00977	4S6 0	4S8 S	2 19	499 8	500 8
0 00407	4SS 8	4S9 7	1 91	500 S	501 0
0 00148	489 6	491 3	1 26	496 5	497 4
			0 977	495 9	497 8
			0 724	495 6	497 0
			0 589	493 5	494 3
			0 316	491 3	493 3
			0 295	492 9	493 5
			0 295	492 2	493 3
			0 138	491 6	492 6
			0 107	488 8	490 7
			0 0851	491 2	493 0
			0 0550	491 2	493 5
			0 0302	490 7	491 6

left it widens rapidly, while to the right it spreads only slowly up to about 570 mμ, beyond which it also widens rapidly   This is precisely what happens to $\Delta\lambda$ for H J in the wavelength discrimination measurements shown in Fig 2 of the preceding paper   Notice that the region between 470 and 570 mμ is one of sharply changing saturation for the colorblind, and that this same region is one of high wavelength
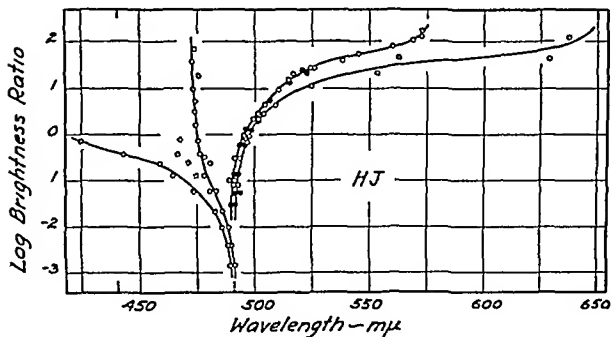
FIG 1   Relative saturation in the spectrum for the protanope H J   Mixtures of 440 mμ and white are to the left of the neutral point   mixtures of 650 mμ and white are to the right   The points give the limits of the band $\Delta\lambda$ which H J matches with each particular mixture   To the right of the neutral point   the mixtures using white of color temperature 5000°K are open circles   while those using the white of the neutral point (491 6 mμ) are solid circles   No difference is apparent in the results   The dotted circles are the ones in parentheses in Table I and were secured by working continuously across the matching band from left to right instead of from each side

discrimination   Since at the neutral point the spectrum appears white and therefore completely unsaturated, the change of the spectrum to either side of the neutral point must be in the direction of increasing saturation, the maximum saturation being reached at the two ends

Some estimate of the degree of saturation at the two ends of the spectrum may be made in terms of the data   Fig 1 shows that to match a just perceptible step from the white of the neutral point, it is

necessary to have a mixture of one part of 440 mμ to about 100 parts of white   This means that H J can tell the difference between white, and white containing about 1 per cent of 440 mμ The least perceptible colorimetric purity of 440 mμ is therefore 0 01 for the colorblind Priest and Brickwedde (1926, see their data in Hecht, 1932) give 0 0022 as the average least perceptible colorimetric purity of 440 mμ for their normal eyes   Assuming the saturation of a spectral color to be inversely proportional to its least perceptible colorimetric purity, then 440 mμ appears about five times as saturated to the normal eye as it does to the colorblind eye

A similar computation may be made for 650 mμ H J can just differentiate the neutral point from a mixture of white containing about 10 per cent of 650 mμ This gives 650 mμ a least perceptible colorimetric purity of about 0 1   Priest and Brickwedde's average for this wavelength is 0 0059 which makes 650 mμ about twenty times as saturated for the normal as for the colorblind

Evidently, the spectrum for the colorblind is reduced in saturation to different degrees in its different parts, so much so that at the neutral point it is completely unsaturated

It is worth pointing out a curious paradox in this connection   From Fig 1 it can be seen that the addition of 10 per cent white to 650 mμ enables H J to match it with about 520 mμ, a really large shift in λ On the other hand, the addition of 10 per cent white to 440 mμ produces hardly any change in the λ which it will match, to match 440 mμ with 470 mμ—a comparatively small shift—requires the addition of 10 parts of white to 1 part of 440 mμ Thus the protanope H J is much more sensitive to the addition of white to 650 mμ than to 440 mμ If the addition of white to a saturated color is more easily perceptible than the same addition to an unsaturated one, this means that 650 appears more saturated to H J than does 440 mμ However, judging by the least perceptible colorimetric purity computation, 440 is about ten times as saturated as 650 mμ

This apparent contradiction is one of interpretation only   It may mean that the colorblind is more sensitive to changes in an unsaturated color than in a saturated one, or that least perceptible colorimetric purity in the colorblind is no measure of saturation, or something different from either   It leaves untouched the main point to be made

from the present measurements, which is that saturation is the basis on which the colorblind discriminates wavelength on either side of the neutral point

IV

Color Mixture as Saturation

We have referred to the gauging of the spectrum with two primaries by the dichromat as color mixture It is important to point out that this is strictly incorrect, that, just as the basis of wavelength discrimination is not hue (that is, color) but saturation, so the basis of spectrum gauging is also not hue but saturation

The dichromat cannot distinguish his neutral point from white Moreover, a specific mixture of two primaries also matches the neutral point Therefore all other mixtures of the two primaries consist of two parts one part made up of the whole of the first primary plus the necessary fraction of the second to make white, and the other part made up of the excess of the second primary To the left of the neutral point, the short wave primary dominates and the variation along the spectrum consists merely in the ratio of its luminosity to that of the associated white made up of the neutral point mixture, while to the right of the neutral point the long wave primary similarly dominates and the spectrum there is matched by the variation in relative amount of primary and white The situation is precisely the same as that just presented in which the spectrum on either side of the neutral point was matched with mixtures of white and either 440 mμ or 650 mμ In the case of the color mixture data the two primaries happen to be 458 7 and 570 0 mμ, and the white is made of a mixture of the two equivalent to the neutral point

If this reasoning is correct, it should be possible to derive from the spectrum gauging (color mixture) data of Table III and Fig 6 of the preceding paper, the amount of white and of excess primary for each mixture data, and a plot of the ratio of primary luminosity to white luminosity for the spectrum should yield a set of curves very similar to Fig 1 in which the relative saturation was measured directly

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[2] Because saturation is so essential a factor in determining λ discrimination for the colorblind it is important to investigate its contribution to similar measurements for the color normal

The computations involve several steps, and may be best explained by an example   Take the primary brightness ratio 22 5/1 whose matching limits for H J are 446 6 and 469 1 mμ   When the bright-
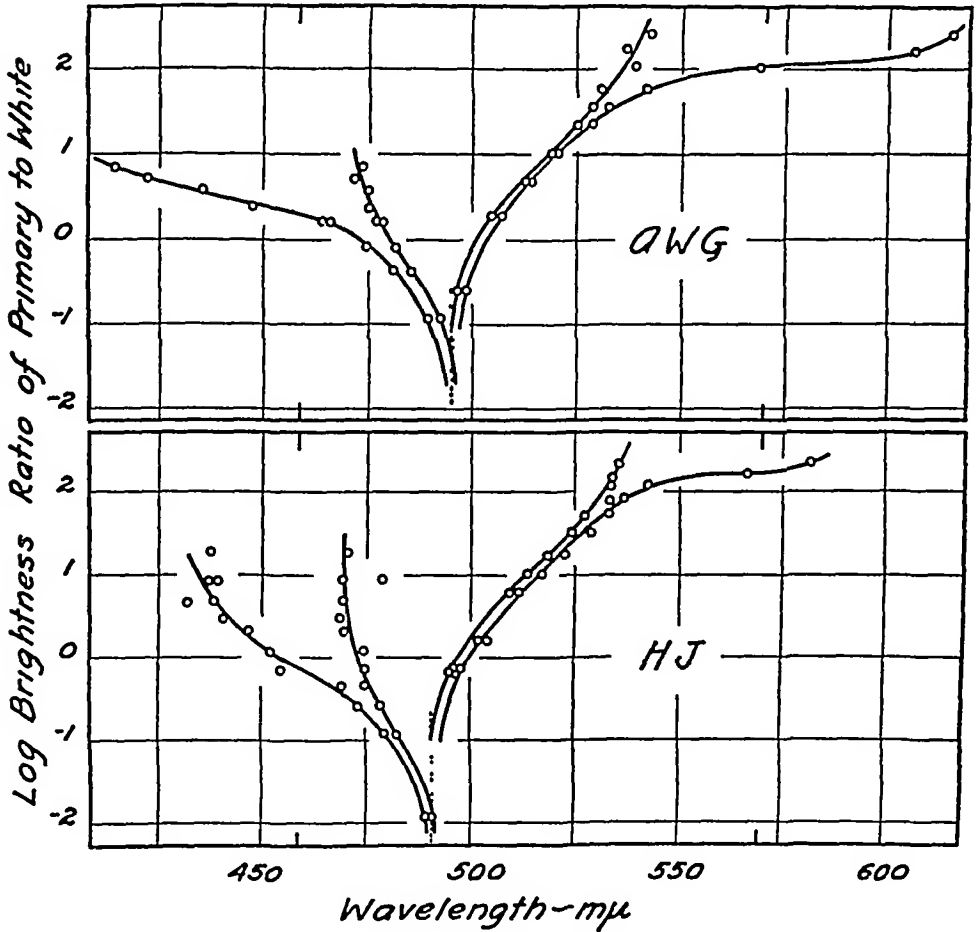
FIG 2 Relative saturation in the spectrum computed from the color mixture data in Table III and Fig 6 of the preceding paper   A W G is a deuteranope, H J a protanope   Compare this figure with Fig 1   Even though the primaries (shown as short vertical lines) in the two figures are different, the computed saturation distribution resembles the measured saturation so strikingly as to leave little doubt of their essential identity

ness of λ570 0 is 1, the total brightness of this mixture is 23 5   The ratio of the same primaries to match the neutral point at 491 6 mμ is 0 063/1, so that when the brightness of λ570 0 is 1 the total bright-

ness is 1 063 and is white   Since the 22 5/1 mixture and the neutral
point mixture of 0 063/1 both contain 1 part of 570 0 mμ, the 22 5/1
mixture therefore contains 1 063 parts of white, while the rest, 22 4
parts, is 570 0 mμ alone   The fraction 22 4/1 063 therefore gives the
ratio of primary to white, and is the information required

We have made these computations for the mixture data of H J
in Table III of the preceding paper, and the results are incorporated
in Fig 2   The results for A W G are also shown in Fig 2   If Fig 2
is compared with Fig 1 showing the saturation distribution as actually
measured, it is obvious that the two are of the same form and appear
ance and yield the same information   The two are not identical
because the primaries used are not the same, in Fig 1 they are 440
and 650 mμ, while in Fig 2 they are 458 7 and 570 0 mμ   But in spite
of this the quantitative similarities are so apparent as to leave scant
doubt that on each side of the neutral point the property which deter-
mines color mixture for the colorblind is the same which determines
wavelength discrimination, and is saturation

It is worth emphasizing this point because it illustrates the fact that
properties of color vision which for the color normal are independent,
become non independent for the colorblind   In the present case,
mixture data and saturation distribution data were secured separately
and by an independent technic   Nevertheless, they turn out to be
related so that one may be derived from the other   Very likely least
perceptible colorimetric purity is a property which is also derivable
from the same data

We began our work with the recognition that the quantitative
determination of only four independent conditions is necessary in
order to describe the color vision of colorblinds and to derive the spec
tral distributions of the basic cone primaries   Considering that five
or six independent conditions were known for color normals, it seemed
a simple matter to find four for colorblinds   However, the four are
not so easily forthcoming for the reason that several conditions which
are independent for the color normal become dependent and identical
for the colorblind

What has come out of the work so far is the recognition of saturation
as the factor which determines the quantitative properties of the color
blind in the spectrum on either side of the neutral point   In terms of

this, protanopes and deuteranopes see the spectrum as white at the neutral point, shading off on the short-wave side to a color (possibly blue) with decreasing amounts of white in it, and on the long-wave side to a color (possibly yellow) also with decreasing amounts of white in it   The spectrum to the dichromat thus appears made up of only two hues, one at each end, these hues gradually become less saturated in the middle portion of the spectrum, reaching complete unsaturation at the neutral point

SUMMARY

1   Wavelength discrimination for the colorblind is entirely determined by saturation differences in the spectrum   From the neutral point to the short-wave end, his spectrum may be completely matched by 440 mμ plus white, to the long-wave end by 650 plus white   The proportion of color to white, hence the relative saturation, changes rapidly in the region of small $\Delta\lambda$ at the center, and slowly in regions of large $\Delta\lambda$ at the ends

2   The data of spectrum gauging with two primaries (color mixture) by the dichromat are shown to contain the saturation distribution in the spectrum for the dichromat   This is because each mixture of primaries may be considered as composed of a mixture which matches white and of an excess of one primary   The data when so computed yield saturation distributions almost identical with those found by direct measurement, and show that on each side of the neutral point the basis of color mixture for the colorblind lies in saturation and not in hue differences

3   To judge by these measurements, the spectrum for the protanope and deuteranope is composed of only two hues, themselves probably of low saturation, situated one at each end   Toward the center these hues decrease still more in saturation until they completely disappear in the white of the neutral point

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STATISTICAL EVALUATION OF SIEVE CONSTANTS IN ULTRAFILTRATION

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It is generally agreed that the principal process involved in ultra filtration is one of sieve action, complicated by adsorption and other effects arising from the extremely large ratio of pore length to pore diameter in all ultrafilter membranes (1-5)

The present paper discusses the rôle of sieving when a monodisperse system is forced through a perfectly isoporous filter membrane, under conditions of "normal" filtration (5), when the primary adsorbing capacity of the membrane has been satisfied, and blocking effects are absent Under such conditions, it has often been implicitly assumed that the disperse phase either passes the filter in undiminished concen tration or is completely retained Thus, when filtration of a disperse system through membranes of different porosities yields filtrates of different concentrations, it appears that there are different sizes of particles in the suspension or solution On this basis, it has been concluded, for example, that certain lyophobic hydrosols (6) and sus pensions of urease (7) and bacteriophage (8) are polydisperse How ever, while some of these systems probably do contain particles of varying sizes, the occurrence of a sieving effect (i e, partial retention of the disperse phase in ultrafiltration) is not a criterion of polydis persion As a matter of fact, sieving occurs in the ultrafiltration of proteins (9) which are known through the results of ultracentrifugal analysis to be virtually monodisperse (10, 11) The semi quantitative theoretical treatment outlined below shows that such sieving in the filtration of a monodisperse system may be anticipated on statistical grounds, on the basis of simple steric limitations in the penetration of filter pores [1]

[1] The interpretation of sieving on a statistical basis has been suggested in a previous paper (5)

Definition of the Sieve Constant

Manegold and Hofmann (12) defined a "sieve constant" (φ) for the process of ultrafiltration, as follows

$$\frac{c_f}{c_s} = \varphi \tag{1}$$

where c_f is the instantaneous concentration of a small sample of filtrate, and c_s is the simultaneous concentration of the filtering solution For complete retention of the solute or disperse phase (i e , for the case of a semi-permeable membrane), $\varphi = 0$, when the solute passes in undiminished concentration, $\varphi = 1$ These authors suggested the possibility that, in certain cases, the sieve constant may be neither 0 nor 1, even when the membrane is isoporous and the solution (or dispersion) monodisperse

Assuming the sieve constant to be independent of c_f, Manegold and Hofmann (12, 13) calculated various expressions for the concentrations of the total filtrate and of the residue at any point in the course of filtration, depending on the manner in which the filtration was carried out (in a closed system, or in a system where the volume of residue was kept up by continuous or intermittent addition of the suspension medium)   Application of these equations to data for the ultrafiltration of colloidal chromium hydroxide (14) indicated that, for membranes of a certain porosity, at least some of the particulate species in the sol filtered with a sieve constant neither 0 nor 1, unless the membranes were heteroporous

We shall employ the definition of Manegold and Hofmann, and proceed to evaluate the sieve constant in terms of microscopical quantities

Scales for Expressing Membrane Porosity

The usual method of calibrating an ultrafilter membrane, by the measurement of the rate of flow of water through it and the application of Poiseuille's law to an assumed structure of cylindrical capillaries of circular cross-section, probably gives a figure for the average pore diameter which is, if anything, too small, especially for a membrane of

low porosity, while the size of the largest particles retained by the membrane is smaller still (4, 15, 5) [2] The continuous scale of porosity grading provided by rate of flow calibrations, calculated in terms of average pore diameter (j), is the most convenient for comparative purposes (5) For statistical evaluation of the sieve constant, however, the pore size will be at first expressed in terms of the diameter effective in filtration (j'), defined equal to the radius of the largest particulate species which absolutely fails to pass the filter under given experimental conditions This diameter j' will be expressed later in terms of the calibrated average pore diameter

Evaluation of the Sieve Constant in Terms of j'

If a monodisperse system of particle diameter J is filtered through isoporous filters of different porosities, the sieve constant for filters of $j' < J$ will be 0 For filters of $j' > J$, φ will lie between 0 and 1, for $j' \gg J$, $\varphi \cong 1$

It is assumed that the membrane structure consists of parallel cylindrical capillaries of circular cross section, a model whose plausibility as a working basis is indicated by studies of water content, diffusion, and conductivity (15, 5), and that, when the membrane is in adsorption equilibrium with the filtering solution, the pore diameter j' effective in the filtration of the disperse phase is also effectively the diameter of the cylinder through which the dispersion medium flows The difference between j and j' is thus attributed to primary adsorption of the disperse phase or of some other capillary active substance (cf (4)) It is further assumed that the solution above the membrane remains homogeneous throughout filtration, local concentration of the disperse phase immediately above the membrane surface being prevented

The filtering solution, considered from a hydrodynamic standpoint, follows streamlines which far above the membrane are uniformly distributed over the membrane area, but in the plane of the membrane surface are concentrated opposite the pore openings and distributed

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[2] These statements refer to ether alcohol collodion membranes and in particular to those of Elford (23, 24 16)

according to Poiseuille flow, i e, at the mouth of any pore, the velocity of flow at a distance ρ from the center is given by

$$v(\rho) = v_1\left(1 - \frac{\rho^2}{r'^2}\right)$$ (2)

where v_1 is the velocity at the center of the pore, and $r' = \frac{1}{2}j'$ The volume of suspension medium entering the pore in time dt is accordingly

$$dV = dt \int_0^{r'} 2\pi\rho v(\rho)d\rho = \frac{\pi v_1 r'^2}{2} dt$$ (3)

A particle of the disperse phase, carried by the flow of suspension medium toward the pore mouth, will have a certain probability of penetrating the latter, depending on how closely it approaches the rim of the opening   For the purposes of this calculation, it is reasonable to take that probability as unity for all particles falling entirely within the opening, that is, those whose centers strike a circle of radius $r' - R$, where $R = \frac{1}{2}J$ The probability of penetration is taken as zero for all those whose centers strike outside such a circle   It is this steric limitation which introduces a statistical sieving   If the velocity of each particle is the hydrodynamical velocity corresponding to the point occupied by its center, Brownian movement being neglected, the number of particles entering the pore in time dt is

$$dn = c_s dt \int_0^{r'-R} 2\pi\rho v(\rho)d\rho = c_s\pi v_1 \left[(r' - R)^2 - \frac{(r' - R)^4}{2r'^2}\right] dt$$ (4)

so that the concentration of the filtrate is

$$c_f = \frac{dn}{dV} = c_s \left[2\left(\frac{r' - R}{r'}\right)^2 - \left(\frac{r' - R}{r'}\right)^4\right]$$

and, replacing radii by diameters, the sieve constant is

$$\varphi = \frac{c_f}{c_s} = 2\left(\frac{j' - J}{j'}\right)^2 - \left(\frac{j' - J}{j'}\right)^4$$ (5)

Expression of the Sieve Constant in Terms of Experimentally Measured Quantities

The end point porosity is defined as the average pore diameter of the most highly porous filter which apparently completely retains the disperse phase (4)   At the end point, equation (5) becomes

$$\varphi = 2\left(\frac{j - j}{j}\right)^2 - \left(\frac{j - j}{j}\right)^4 \tag{6}$$

where φ_e is the smallest relative concentration of the disperse phase which can be detected by the analytical methods employed, and j'_e is the end point porosity measured on the filtration effective scale (only very slightly greater than J)   The end point porosity in terms of the calibrated average pore diameter, j_e, may be substantially greater than J, and the relationship between the two may be determined for a given type of suspension by filtration of suspensions of known particle size (4, 5), to evaluate an experimental correction factor

$$q = J/j \tag{7}$$

The difference between j_e and j'_e, attributed to primary adsorption of the disperse phase (or of a capillary active substance) within the pores, is now assumed to represent a constant difference between the two porosity scales, thus

$$j - j = j - j = j - \frac{J}{1 - \sqrt{1 - \sqrt{1 - \varphi}}} \qquad \text{(by equation (6))}$$

and

$$j = j - j + Jf \tag{8}$$

where

$$f = \frac{1}{1 - \sqrt{1 - \sqrt{1 - \varphi}}}$$

Substituting (8) into (5) we obtain

$$\varphi = 2\left\{\frac{j - j + J(f - 1)}{j - j + Jf}\right\}^2 - \left\{\frac{j - j + J(f - 1)}{j - j + Jf}\right\}^4 \tag{9}$$

in terms of experimentally measured quantities—the calibrated porosity j, the calibrated end-point porosity j_e, and the particle diameter J

Factors Ignored in the Above Treatment

It is possible that the above criterion for penetration of a pore is too restricted, a particle may strike the surface of the membrane so that it does not quite fall entirely within the pore opening, and yet may glance off the rim and be carried into the pore by the flow of dispersion medium   Such a particle, however, would probably be delayed by a drag from the membrane surface, so that the resulting enrichment of the filtrate would be minimized   Another factor in the penetration of pore openings which has not been considered is the influence of the electrical charges of membrane and disperse particles   Probably, however, the most serious objection to the calculations outlined above lies in the fact that penetration of a particle into a pore does not assure its emergence at the bottom of the membrane   The ratio of pore length to pore diameter is seldom less than a thousand to one, and, although the tortuosity of pores as pictured by some authors has perhaps been exaggerated (15, 5), it is likely that many particles become lodged in the membrane in the course of filtration   From this standpoint, values of the sieve constant calculated from equation (9) may be too large   The discrepancy, however, should be the least in the range of sieve constants approaching unity   This is found to be the case when equation (9) is compared with experimental data for ultrafiltration of viruses (see below)

Comparison of Equation (9) with Experimental Data

For suitable experimental data with which equation (9) may be compared, reference is made to the work of Elford and collaborators, who have employed graded collodion membranes of a high degree of isoporosity in ultrafiltration studies under standard conditions of normal filtration   Their data give the maximum relative concentration of filtrate, u_{max}, as a function of the membrane porosity j (the "end-point curve" (9) or "filtrability curve" (16))   While u differs somewhat from φ, being the ratio of filtrate concentration to the initial, rather than the instantaneous, concentration of filtering solu-

tion, the curves for u_{max} (experimental) and φ (equation (9)) should be qualitatively comparable   The experimental u_{max} should in general be higher than φ, since the residue becomes concentrated in the course of filtration, this concentrating effect is, however, opposed by the primary adsorption of the membrane

Comparisons are made for the ultrafiltration of suspensions of horse serum albumin in water at two pH's (9), of hemocyanin (*Helix*) in Hartley's broth (17), and foot and mouth disease virus in broth (18) For the proteins, the values of J are taken from Svedberg's data (10), on the basis of spherical particles (ignoring the slight anisodimension ality in the case of serum albumin), the value for foot and mouth

TABLE I

Constants Employed for Calculation of Theoretical Curves

	j	J	e	f
Serum albumin dispersed in water pH 8 8	11 mμ	5 4 mμ	0 01	1 08
Serum albumin dispersed in water pH 4 1	12 mμ	5 4 mμ	0 01	1 08
Hemocyanin (*Helix*) dispersed in Hartley s broth pH 7 3	55 mμ	24 mμ	0 01	1 08
Foot and mouth disease virus dispersed in Hartley s broth pH 7 6	25 mμ	10 mμ*	10^{-4}	1 00

* Calculated by application of the correction factor $q = 0 41$ (reference (5))

disease virus is calculated by equation (7) (*cf* (4, 5))   The constants required for equation (9) are summarized in Table I   The experi mental and theoretical curves are compared in Fig 1

Serum Albumin —The theoretical curves for serum albumin, which do not differ much from each other, fall between the experimental curves for pH 8 8 and pH 4 1   This situation might seem attributable to the effect of the charge of the protein (negative in one case and positive in the other), which was not taken into account in the theo retical considerations   However, such an interpretation would mean an enhanced probability of penetration in the case of negatively charged particles, whereas much experimental evidence (19, 20) shows that collodion membranes are *less* readily penetrated by negatively than by positively charged particles   The fact that the experimental

curve for pH 8 8 is high is probably due to the nature of the comparison between u_{max} and φ, as explained above   The experimental curve for pH 4 1 is low, probably owing to the fact that filtration at this pH does not proceed under quite "normal" conditions, some blocking occurring (9)   The slight degree of anisodimensionality of the serum albumin molecule probably has little influence on the statistics of pore penetration

Hemocyanin (Helix) —The agreement between theoretical and experimental curves for hemocyanin, which is practically within
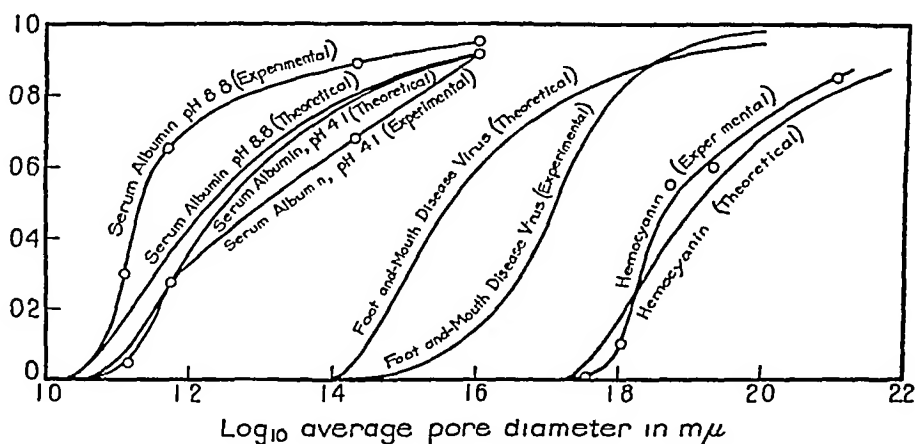
FIG 1 Comparisons of theoretical and experimental filtrability curves   The sieve constant (theoretical) and maximum relative concentration of filtrate (experimental) are plotted against the logarithm of calibrated average pore diameter in $m\mu$

experimental error, is probably due to a counterbalancing of the two factors mentioned in the preceding paragraph   The filtration of hemocyanin was, in fact, not quite "normal" (17)

Foot-and-Mouth Disease Virus —The theoretical curve for foot-and-mouth disease virus rises much faster from the end-point than does the experimental curve   This effect is still more marked for other viruses and bacteriophages, so that it is impossible to reconcile their end-point curves with curves calculated by equation (9)   Thus, for membranes of porosity only slightly above the end-point value, the probability for transmission of virus is apparently much lower than that derived from the simple steric considerations of equation (4)

This situation may be ascribed to the difficulty of saturating the primary adsorbing capacity of the membrane in dilute virus suspensions (4, 5)   However, the general agreement of the end point curve for foot and mouth disease with the theoretical curve is sufficient to suggest that the shape of the former may be due to statistical sieving alone, without the necessity of postulating the existence of aggregates held back at high porosities, or of a certain degree of heteroporosity in the filters employed

DISCUSSION

This formulation of statistical sieving supports the viewpoint that the appearance of sieve action is not a criterion for heterodispersion in the system filtered nor heteroporosity in the filters employed Heterodispersion may be demonstrated by the appearance of sieving over a much wider range of porosities than is the case for a monodisperse system (as was shown by Grabar and Riegert (21) for urease), or by ultrafiltration of fractions separated by centrifugation or previous filtrations (as was shown by Bechhold (22) for a silver sol)

The applicability of equation (9), considering the excessive simplifications employed in its derivation, lends encouragement to the possibility of attacking the problems of osmosis and penetration on a microscopical basis

SUMMARY

The partial retention of the disperse phase in the ultrafiltration of a monodisperse system through an isoporous filter is interpreted on a statistical basis, and a simple expression for the sieve constant is evaluated in terms of the calibrated membrane porosity and the particle size   Curves calculated from this expression are in reasonable agreement with experimental data for the ultrafiltration of serum albumin, hemocyanin (*Helix*), and foot and mouth disease virus

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ELECTRIC IMPEDANCE OF INJURED AND SENSITIZED RED BLOOD CORPUSCLES

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INTRODUCTION

In a recent paper on the electric impedance of hemolyzed suspensions of mammalian erythrocytes (Fricke and Curtis, 1935 a), it was shown that even after a red cell is hemolyzed it still offers a high resistance to the passage of an electric current, or, in other words, that the membrane is still relatively impermeable It was further shown that for normal cells the membrane capacity and resistivity at low frequency increase only slightly with decreasing frequency, while they change quite rapidly with the frequency in the hemolyzed suspensions Moreover, in the case of a chemical lysin such as saponin the increase becomes more marked as stromatolysis proceeds

It was pointed out that a polarization capacity due to selective ionic permeability could account for this increase and the more marked increase in the case of the hemolyzed cells could be explained as due to an increased permeability However, another explanation was advanced, namely, that the increase might be due to a polarization of the current passing through the double layer, such as has been observed in aqueous suspensions of non conducting particles like powdered glass or paraffin oil (Fricke and Curtis, 1935 b) On this hypothesis, the change on hemolysis would be due to some sort of a change in the double layer The former of these two possibilities seemed the most likely

Thus it appeared as though the electrical method presented a new way to measure changes in the cell membrane In order to test this, it was decided to make a series of measurements on red cell suspensions which had been treated in various ways to produce changes in the

membrane, to see if the low frequency variation of capacity is affected by any of these changes

EXPERIMENTAL PROCEDURE

The method of measurement was the same as that previously described (Fricke and Curtis, 1935a) except that the measuring cells were of a different size   The ones used were 20 cm long and had a cross-sectional area of 1 3 cm ^{2} The method consists essentially in using a Wheatstone bridge to indicate the electrical equivalence of a measuring cell filled with the suspension and an exactly similar cell filled with a salt solution with a variable air condenser in parallel to it   The resistance of the suspension is then the resistance of the salt solution and its capacity is the capacity of the condenser plus the static capacity of the comparison cell   The greatest single error in such measurements at low frequencies is due to electrode polarization   Because of this and other errors which will be discussed in a subsequent paper, it has not been found possible to make these measurements with sufficient accuracy for the present purpose at frequencies below 2 kilocycles per second

Rabbit blood or washed rabbit cells was used in all the experiments   To obtain lecithinated cells, just enough lecithin was added to the plasma to make the cells completely spherical   The complete change of shape presumably corresponds to some important change at the cell surface (Ponder, 1936)   In the case of systems containing glucose, the cells were suspended in 5 6 per cent glucose, their permeability is presumably modified in such a medium, for they lose salts (Joel, 1915, Kerr, 1929), and they may eventually hemolyze (Yeager, 1929)   In the case of saponin, half as much saponin was added as would cause hemolysis in 30 minutes   In the colloidal silicic acid, amboceptor, and tannic acid systems, enough sensitizing agent was added in each case to sensitize the cells completely to guinea pig complement, but not enough to cause agglutination   It is not known whether permeability changes are associated with sensitization or not, but the nature of the cell surface must be considerably modified   In no case was there any hemolysis at the end of the experiment

RESULTS

The results are shown in Fig 1, the membrane capacity in μf per cm ^{2} being plotted against the frequency   Since the absolute value of the membrane capacity cannot be measured with as great an accuracy as the variation of this capacity with the frequency, and since we are interested primarily in this variation, the capacity values have arbitrarily been made to agree at 32 kilocycles   There is no discernible systematic variation in the absolute values

It will be seen that the largest variation is exhibited by the leci-

thinated corpuscles, but even here it is somewhat doubtful if the experimental accuracy is good enough to enable us to say definitely that the increase is greater than that for normal cells The dotted curve is a reproduction of a typical curve obtained for a suspension of hemolyzed red cells, and is included to give an idea of the relatively
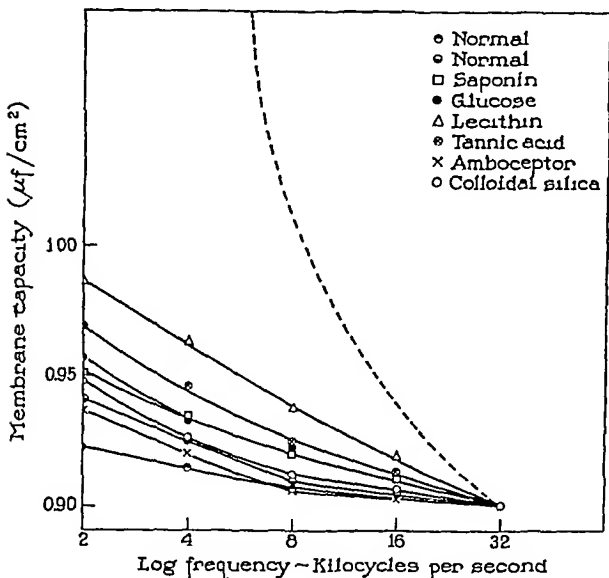
FIG 1 Membrane capacity vs log frequency for normal, injured, and sensitized red cells The dotted curve represents hemolyzed red cells

enormous increases encountered in these cases, and to show that the present increases do not in any way approach them

It was not possible in all cases to measure the variation of resistivity with frequency, but where such measurements were made it was found that, within the experimental error, the variation was the same as for normal cells

DISCUSSION

The results show conclusively that there is no marked increase in the frequency variation of the membrane capacity of rabbit red cells when they are "injured" or sensitized up to the point of hemolysis or agglutination   While it is not possible to state exactly what sort of changes in the membrane have taken place in each case, it can certainly be said that marked changes in permeability have taken place at least in the cases of glucose and saponin, and in all cases the membrane is far from being unchanged   This, therefore, is not a sensitive method for measuring changes in membrane properties, although a prolonged and careful investigation might show a statistical difference between normal and injured cells

The result may mean that the form of the frequency variation is an extremely insensitive measure of permeability and other membrane changes, and capable only of disclosing the very great changes associated with hemolysis, or it may mean that the change in the frequency variation at low frequencies has nothing to do with permeability, as has been already pointed out

The author is indebted to Drs Eric Ponder and Hugo Fricke of this Laboratory for assistance and cooperation in this work, and to Dr L Reiner of Burroughs Wellcome & Co for preparing the anti-rabbit amboceptor

SUMMARY

On the basis of previous work on the electrical properties of hemolyzed red cells, it might be supposed that the variation of the capacity with frequency at low frequencies is an indication of membrane permeability   To test this, rabbit red cells were subjected to treatment with lecithin, tannic acid, glucose, saponin, amboceptor, and colloidal silicic acid, each in sub-lytic doses   No change in any of the electrical properties of any of the suspensions could be detected   The result may mean that the form of the frequency variation is an extremely insensitive measure of permeability and other membrane changes, and capable only of disclosing the very great changes associated with hemolysis, or it may mean that the change in the frequency variation at low frequencies has nothing to do with permeability

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ANALYSIS OF THE GEOTROPIC ORIENTATION OF YOUNG RATS X

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I

The investigation of the inheritance of physiological characters requires that aspects of the performance of individual organisms be measured as quantitative functions of relevant variables The possibility of demonstrating in this way the heritable character of elements of behavior has a very special importance   We have been led to an examination of this matter (Crozier, 1929, Crozier and Pincus, 1929-30a, Crozier and Pincus, 1931-32d) partly from the standpoint that, in the first place, a peculiarly significant procedure is thus made available for the testing of quantitative formulations of behavior in particular instances   It has also been pointed out that a type of procedure is thus illustrated which makes possible the recognition and the genetic use of traits otherwise obscure or unrecognized, and which may be taken to provide a schema for the interpretation of breeding results which could otherwise easily be regarded as confused or theoretically unmanageable   An important part of this procedure involves the analytical use of measures of the capacity of the organism to vary the expression of the estimated performance under controlled, measured inciting conditions

The specific element of performance extensively utilized has been the geotropic orientation of young rats of several different inbred lines (Crozier and Pincus, 1926, 1926-27, 1929-30a, etc)   The extent of upward orientation (angle θ) during creeping upon an inclined surface is a characteristic function of the slope of surface (angle α) for each inbred line   Three of these lines have been employed in genetic tests of the assumptions (1) that the quantitative differences observed are "real," and (2) that the forms of the relationships between θ and α

are in a biological sense significantly and properly expressed by the curves drawn to describe them   The results obtained from the breed-ing of lines A and K have already been considered (Crozier and Pincus, 1929–30 a, b), the outcome of crossing lines A and B has also been dealt with so far as concerns the F_1 generation and the backcross of F_1 with B (Crozier and Pincus, 1931–32 d)   We now discuss the result of back-crossing $F_{1(A \times B)}$ with line A

In none of these tests could the quantitative features of the outcome be predicted with certainty, the situation is essentially complex, and the whole procedure is to a degree still in an exploratory stage of de-velopment   Yet the body of results to the present has given a most encouragingly consistent picture   This internal consistency is perhaps its strongest feature   The nature of the case investigated has placed physical limitations upon the numbers of individuals it has been pos-sible to examine, the present paper, for example, has been based upon the consideration of approximately 10,000 orientation angles   The interpretation of the variability of performance has thus a special importance for the verification of the classification of individuals assumed to represent diverse classes of segregates   In any case, the crucial thing about the present material is that it was predicted (Crozier and Pincus, 1931–32 d) that in the backcross $F_{1(A \times B)}$ with B the progeny were expected to be all of one type, and essentially indis-tinguishable from the B stock as regards θ vs α, since the F_1 individuals appeared to show dominance of the B condition, whereas with $F_1 \times A$ the progeny should show marked diversity among the individual θ—α curves   The data demonstrate abundantly that this expectation is realized   There is also obtained a very striking illustration of the quantitatively reproducible character of specific indices of variability

<div align="center">II</div>

Young rats of our races A and B (Crozier and Pincus, 1927–28, 1929–30 a, 1931–32 a, b, d) give characteristic, reproducible relation-ships between angular extent of upward orientation (θ) and inclination of substratum (α) during geotropic progression   The F_1 individuals from cross-matings of these races show in quantitative features of their geotropic behavior an essential dominance of the type of per-formance given by the B parents   The diagrammatic quality of this

dominance is only slightly upset by a kind of "heterosis" effect which we have interpreted (Crozier and Pincus, 1931–32 e) as due to a particular sort of disharmony of developmental processes in the F_1 rats The relationship is further complicated, however, by disturbances of the *variability* of the orientation angles, although these are not pronounced, the total observed variation of θ is not affected, but the variability uncontrolled by the magnitude of $\sin \alpha$ is increased, so that the percentage modifiable variation is reduced This effect disappears in the *adult* Γ_1's (Crozier and Pincus, 1935–36) These relatively minor differences between the young F_1 individuals and B rats largely disappear in the backcross generation $(\Gamma_1 \times B)$ so that these backcross rats are distinguishable from their B grandparents only by a slightly lower percentage of modifiable variation of θ This is the result to be expected if the increase of the "proportion of the variation of θ not a function of $\sin \alpha$" is determined by the influence of 'modifiers' not directly linked with the determination of θ as related to α, and perhaps not primarily of a genetic character The $(F_1 \times B)$ generation should then show, as a group, a percentage modifiable variation intermediate between that for Γ_1 and for B, and this was found In $(\Gamma_1 \times B)$ rats the slight distortion of the fundamental B curve (θ vs log $\sin \alpha$) which the F_1 individuals show, is not certainly detectable This was attributed to the practical suppression of the "developmental disharmony" which in Γ_1 rats makes it appear as if a posterior region of the body grows to a certain extent faster than the multiplication of the correlated complement of tension receptor units in the posterior legs (Crozier and Pincus, 1931–32 e)

When the results of the opposite backcross $(F_1 \times A)$ are to be considered, the situation is expected to be much more involved We have pointed out that the contrast to be looked for in the behavior of $(F_1 \times A)$ and $(F_1 \times B)$ rats supplies a searching test of the competence of the whole analysis (*cf* Crozier and Pincus, 1931–32 e) The A and the F_1 and B lines show differences as regards (1) threshold $\sin \alpha$, (2) θ at threshold, (3) the θ vs α curve, at all points, the measures of variability (4) V N and (5) V N $_{\sin \alpha}$, (6) total observable variability of θ, and (7) percentage modifiable variation

We have indicated that the threshold $\sin \alpha$ appears to be connected with the shape and dimensions of the population curve for sensory

thresholds in "receptor group" *1*, but that there is no apparent necessary relationship between *sin α threshold*, in various races of rats, and *θ* at this threshold, whereas total variation of *θ* is determined by the total number of receptor units (*cf* Crozier and Pincus, 1931–32*e*, Crozier, 1935)   We have no reason to expect that the magnitude of *θ* at threshold sin *α* will be determined in the same way as threshold sin *α*, and we may well expect that it should be of quite independent nature through its involvement with central nervous properties   Theoretically, the magnitude of sin *α* normally appearing as threshold slope should measure the force necessary to excite tension receptors in the legs to an extent such that an oriented response appears   The magnitude of the orientation angle presumably measures the corresponding excitation, if orientation means that tension-excitation is sensorially equivalent (within a threshold difference) on the two sides of the body   This is thoroughly substantiated by suitable experimental modification of the units in which sin *α*, and the response, are measured (Crozier and Pincus, 1931–32 *d*)   The amount of excitation at threshold slope of surface, on the other hand, will depend upon the "central resistance" which this form of excitation must overcome before it can dominate behavior and lead to orientation   The "amount of excitation" just below the threshold of course appears graphically as *zero*, since it cannot be expressed in the coordinates used Hence the properties of the response which interest us may be expressed by a curve which lies at one or another position, vertically, on the *θ—α* grid, depending upon conditions affecting the threshold response, although the *slopes* of the *θ—α* relationship (indicating the way in which increasing sin *α* brings into action additional tension receptor units) may or may not remain the same   The fact that intercepts and slopes of the *θ—α* relationship differed independently in sundry pure lines of rats, in our initial experiments led us to suspect that at least two *kinds* of genetic differences must be concerned in the characterization of distinct types of curves of geotropic response (Crozier and Pincus, 1929–30 *a*)

We have been concerned primarily with a method of discovering whether it is possible to demonstrate, by independent, non-ambiguous criteria, that quantitative formulations of the performance of a biological system are valid, competent, real   Two steps in this procedure

are (1) the reduction of the variation of performance to rational meas urement and dissection, (2) the testing of the biological reality of equations expressing performance through the *genetic* behavior of their characteristic constants   The two steps are interrelated, and require the use of estimates of the functional dependence of performance upon a known, measured, governing variable   It has been emphasized (Crozier and Pincus, 1929–30 a, 1931–32 a, c, 1932) that the converse use of such systems of relationships, as a means of genetic analysis, is not merely desirable, but necessary, although we cannot expect to encounter at first situations entirely simple

From breeding experiments with races A and K (Crozier and Pincus, 1929–30 b) we know that the three main groups of tension receptors implicated in the orientation of geotropic creeping, as defined by the frequency distributions of their receptor threshold, are inherited inde pendently   Two of these groups (A_1, A_2) were dominant over their K homologs (K_1, K_2), the third group (A_3) being recessive to K_3 But B_1, B_2, B_3 are dominant over A_1, A_2, A_3 This is not alone attested by the correspondence of the θ—α curves for B, F_1, and ($F_1 \times B$) as regards the absolute magnitudes of θ, but by the threshold slope for significant orientation and by the orders of magnitude of the indices of variation of θ The way in which the two latter quantities appear to be determined by the form of the frequency distributions of thresh olds for excitation of the receptor units, and by the total number of these units, has been given preliminary consideration in earlier papers In the cross $A \times B$ it was recognized, however (Crozier and Pincus, 1931–32 c), that there appeared in F_1 the effect of influences decreasing the proportion of the modifiable variability of θ On this general basis we have reason to expect that the offspring of matings of F_1 individuals with A rats will be of several distinct classes at least, in which all possible combinations of ' large" and "small" assemblages of receptor unit groups 1, 2, and 3 will be phenotypically exhibited, with equal frequencies of occurrence   This is without making allowance for any possible effect of differences in the threshold slope of surface for geo tropic orientation, or of orientations at threshold slopes

We have seen reason to regard the threshold slope for orientation as signifying the magnitude of gravitational pull ($\sin \alpha$) adequate to enforce significant excitation to an extent which dominates behavior,

in the sense that statistically recognizable orientation is apparent, and that this threshold slope is determined by (1) the *form* and *area* of the group *1* receptor frequency distribution and (2) the magnitude of the "central" threshold for geotropic response (Crozier and Pincus, 1931–32 *c*, *d*)   If the latter element in the situation is governed by independent factors which may or may not be subject to unitary inheritance, and may be connected or not in a genetic or a developmental way with the sensory units given by the method of estimation we necessarily employ, a very confused condition may result in the population with which we now deal   We might then encounter some ten or more distinct classes of individuals, since a "*B*" group *1* might or might not be associated with an "*A*" —or a non-*B*—threshold α The fact that threshold α increases with increasing age of individual, in a pure line, might make it desirable to use a still further coordinate, age, for adequate analysis of the curves   The argument against the occurrence of this particular phenomenon, however, is based in part upon the fact that the extreme upper value for θ in the *B* stock is, at α = 20°, about θ = 62°, the extreme lower θ for *A* rats being θ = 51°, this gives a zone, 51° to 62°, within which, in fact, all the determinations for (F₁ × *A*) individuals are found to fall at this slope of surface

The converse possibility, nevertheless, does exist   A rat with *A* group *1* and *B* threshold θ would be expected to show, at α = 20°, a much lower orientation angle than θ = 51° ± 1°, since we have reason to hold that excitation of sense organs below threshold θ does not affect the magnitudes of higher angles of orientation (*cf* Crozier and Pincus, 1931–32 *d*), *none* of the actual determinations are below this limit   At the other end of the θ—α curve we would find, on the basis of this possibility, that there should occur *B* type individuals with *A* threshold θ, this combination would result, it must be presumed, in rats with θ at α = 70° above the θ = 84° ± 0 5° characteristic of the *B* stock   In fact the entire lot of determinations of θ at α = 70° for 41 (F₁ × *A*) individuals examined range from θ = 76 3° ± 1 09° to 85 9° ± 0 53°, while the limits given by the performance of *A* and *B* grandparents are 79° to 84°   The occurrence of occasional low individual mean θ's at α = 70°, through slipping, is understandable, and is recognizable by the behavior of σθ, the notable fact is that no values are greatly above that for the *B* race   The test is more rigorous at

$\alpha = 55°$ The range expected in the absence of any heritable "thresh-old factor" is from a little below $\theta = 78° \pm 0\ 8°$ to somewhat above $82° \pm 0\ 5°$, that found is from $\theta = 76\ 5° \pm 1°$ to $83° \pm 0\ 9°$ Remembering that the θ's for $(F_1 \times A)$ rats are for individual animals, the correspondence is extraordinarily good

We may then proceed to an attempted analysis of the individual $\theta—\alpha$ curves obtained for $(F_1 \times A)$, with the following possibilities in view that the receptor groups *1, 2, 3* should be inherited alternatively as B type or A, with B dominant, that a B_1 type curve should show the threshold α and the mean θ characteristic of that type, that an A_1 type curve should show the threshold α characteristic of that type of curve, with or without an *increase* in the mean θ as compared with that for pure line A individuals, and that differences in the *variability* of θ should appear in both B_1 and A_1 type rats, such that some of each should exhibit a greater proportion of uncontrollable variation of θ in the low α portion of the $\theta—\alpha$ graph The "disharmony effect" (Crozier and Pincus, 1931–32 d) we do not expect to encounter in pronounced form, for reasons earlier discussed These expectations are based directly upon the immediately foregoing considerations, and do not exclude the possibility of other complications

A relatively large number of cases would be required for final demonstration of the details of a workable factorial basis for such a scheme, or of its limitations, since some 10 or 12 classes of $(F_1 \times A)$ individuals would be expected

Our primary purpose now will be served if we can succeed in obtaining demonstration that genetic segregation of the factors responsible for the magnitudes of θ in A and B stocks does indeed occur The possibility of such demonstration is at once given by the facts of observation A very considerable diversity of orientation angles is obtained with the $(F_1 \times A)$ individuals, a state of affairs quite unlike that in the $(F_1 \times B)$ population To this extent the expectation of a pronounced difference between the $(F_1 \times B)$ and the $(F_1 \times A)$ backcross is thoroughly realized We regard this as a very striking phenomenon, and as one incomprehensible save upon some such basis of theory as that we have been encouraged to use, it excludes the possibility of incompetent observation

Orientation data were obtained from forty-one individuals, in mat

ings $F_1 \times A$ and $A \times F_1$, which are summarized in Table I The procedure has been described (Crozier and Pincus, 1931–32 *a*)

The fact that the constancy of $\Delta(P\ E_\theta/\theta)/\Delta\theta$ is not affected by experimental modification of the conditions of excitation, as by the carrying of additional loads (Crozier and Pincus, 1931–32 *d*), makes it possible to deal with this index of variation ($V\ N_\theta$) as a check upon the classification of individuals Such use is predicated upon the finding that the relative variation of θ is controlled by the conditions of excitation in the same manner as is the extent of the orientation itself (Crozier and Pincus, 1926–27 *b*), and that the two are organically interdependent (Crozier and Pincus, 1931–32 *d*) It is to be remembered, however, that we are here dealing with data from single individuals, not with figures applicable to litters, and that P E of P E$_{\theta_1}$ is thus relatively high ($n = 20$ at each α), so that it may be rather difficult to determine the slope of the line connecting P E$_\theta/\theta$ with θ, in any one instance, owing to the greater scatter of θ's at low slopes of surface Moreover, we have seen reason to believe (Crozier and Pincus, 1931–32 *e*) that influences affecting *variation* of θ, without materially influencing mean θ, may be involved in the cross-breeding result

III

The classification of individuals arising in the backcross progeny of matings between F_1 and race A presents a number of difficulties In the light of previous experiments we might assume that the rats possessing a (dominant) B group 1 of excitation elements should orient at $\alpha = 15°$ (*cf* Crozier and Pincus, 1931–32 *a*), those exhibiting A_1 not until about $\alpha = 20°$ We have already found, however, that neither the threshold slope of surface nor the extent of response (θ) at this threshold gives a thoroughly adequate basis for comparison of geotropic reactiveness among diverse pure lines (Crozier and Pincus, 1931–32 *b*) We must in addition expect to encounter possible slight shifts of the maxima in the distribution curves for excitation groups 1, 2, and 3 (*cf* Crozier and Pincus, 1929–30 *b*), as well as slight shifts of the θ—α curve upward or downward on the θ axis, the latter effect is to some extent apparent in pure lines, with rats of fixed age, particularly in comparing different litters And with advancing growth

(at 24 days, or thereabouts, in race A, for example), the apparent threshold α is increased   A technical difficulty possibly inheres in the fact that in taking observations at low slopes of surface the tendency inevitably is to impute orientation if possible, the probable errors of the mean θ s of course serve to check this, the fact that the observed threshold α varies a little from time to time is given added significance by the finding that threshold α for significant upward orientation is easily modified by certain experimental treatments—for example, by suitable injection of caffein solution subcutaneously, added weights (Crozier and Pincus, 1929–30 b), or the injection of adrenin (Crozier

TABLE I

Mean angles of orientation (θ) for all individuals in the backcross generation ($F_1 \times A$) as a function of inclination of surface (α)   Average orientation angles for lines A and B with these values of α are included for comparison   See Fig 1

α degrees	θ degrees		
	A ○	($F_1 \times A$) ●	B ◑
15		54 4	55 2
20	52 8	58 2	60 4
25	57 3	61 5	64 5
30	60 6	64 4	68 5
35	63 9	70 0	72 3
45	71 7	75 0	77 7
55	77 5	79 5	82 0
70	79 8	81 3	83 7

and Pincus, 1932–33 a, etc)   The final test for segregation of B vs A groups 1, 2, 3 must be made independently of threshold effects, and must be based upon the shapes of the individual curves, but in applying this test it must be remembered that the general slope of the θ—α curve may be affected by the initial amplitudes of orientation— since a high initial θ may necessarily require a lower tilt of the θ—log sin α curve when the upper limit of maximum orientation is relatively fixed   When diverse races of rats are compared (cf Crozier and Pincus, 1926–27 b, 1931–32 a, etc)   there is, however, no necessary correlation between lowness of threshold θ and steepness of the θ—α curve The presence with some individuals of the increased scatter of mean

θ's at low α's (*cf* Crozier and Pincus, 1931–32 *e*), makes it necessary to employ all possible criteria

The mean angles of orientation (θ) for all ($F_1 \times A$) individuals are given in Table I   The ($F_1 \times B$) rats (Crozier and Pincus, 1931–32 *e*) agree quantitatively with grandparental line *B*, but the average values
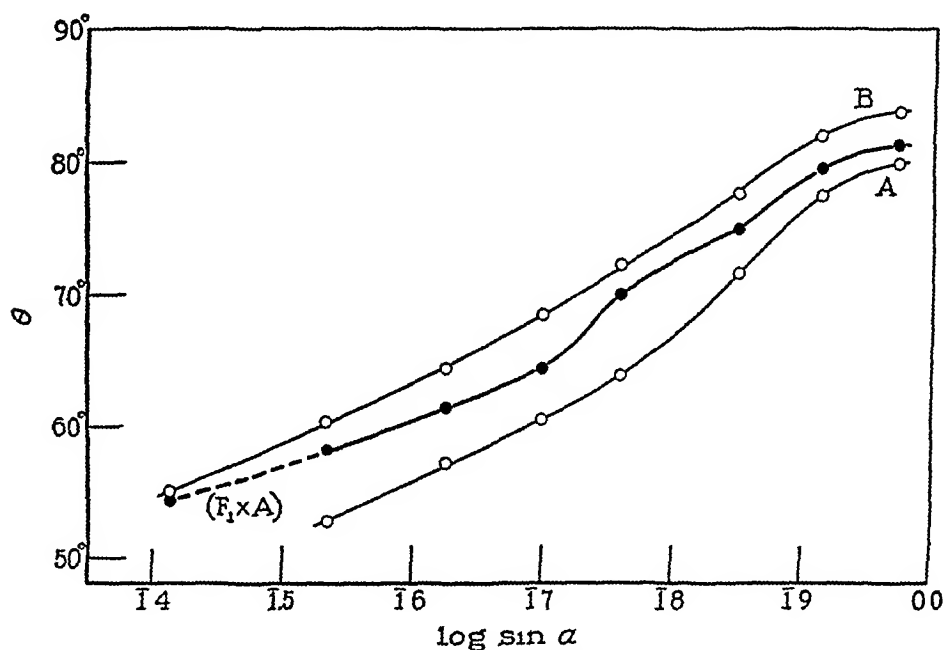
FIG 1 Orientation angle (θ) as a function of inclination of surface (log sin α) for young rats of lines *A* and *B*, based on weighted means of various series of observations (data for several slopes of surface in single series of observations are omitted), and for the total population of young rats produced in the backcross generation $F_{1(A \times B)} \times A$ The backcross generation includes a number of individuals (see text) which did not orient at $\alpha = 15°$, the curve between $\alpha = 15°$ and $\alpha = 20°$ is accordingly dashed   In a general way the curve for the lumped data of the backcross generation is intermediate in position between that for the two rat grandparental strains, its form indicates, in the light of experience with various races of rats, that the $F_1 \times A$ group is heterogeneous

for ($F_1 \times A$) are strictly intermediate between those found for *A* and for *B* pure lines   The value at $\alpha = 15°$ ought to be, as it is, if anything a little lower than for *B*, since we do not expect A_1 type rats to orient at $\alpha = 15°$, but only at about $\alpha = 20°$ Fig 1 shows that the curve has a character differing from that for pure lines *A* and *B*, with a signifi-

cant irregularity at $\theta = 70°$, this is equally demonstrated in the
behavior of the mean variation of θ, for lines A and B, P E, is a declin
ing rectilinear function of sin α, for the combined data (forty one indi
viduals) in $(F_1 \times A)$ P E, at $\alpha = 35°$ is "too high," and the curve
(Fig 2) is broken, the *relative* variation of θ is also too high   Precisely
the same sorts of indications are given by the averaged measurements
for the members of single litters, the means agree with those given for
the total $(F_1 \times A)$ population, and the relative and the absolute varia
tions of θ show corresponding discontinuities
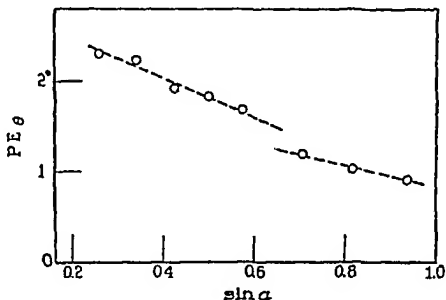
FIG 2 P E θ as a function of sin α for the lumped observations from rats
produced in the backcross $F_1 \times A$ as in the case of the $F_1 \times A$ curve in Fig 1,
the discontinuity indicates a population which is heterogeneous as concerns the
manner of dependence of the probable error of mean θ upon sin α

These facts all indicate (1) that the $(F_1 \times A)$ individuals differ
from those produced by the backcross $(F_1 \times B)$, and (2) that the
$(F_1 \times A)$ population is heterogeneous
On the most direct assumptions we expect to find only some of the
$(F_1 \times A)$ rats orienting at $\alpha = 15°$, and mean θ at $\alpha = 15°$ unimodally
distributed, whereas all the rats should orient at $\alpha = 20°$, with mean
θ's not distributed unimodally, and the P E 's of the mean θ's should
show similar distributions (cf Crozier and Pincus, 1931–32 a, b)
Twenty one oriented at $\alpha = 15°$, three others were rather "doubtful,"
but gave no definite and reliable evidence of orientation below $\alpha = 20°$

All the rats oriented typically at $\alpha = 20°$ It will be noted that this is just about the proportion to be expected if lower threshold α is associated with B_1, higher threshold α with A_1

In the absence of complications affecting threshold slope of surface, such as we have already referred to, we would expect a slope $\alpha = 15°$
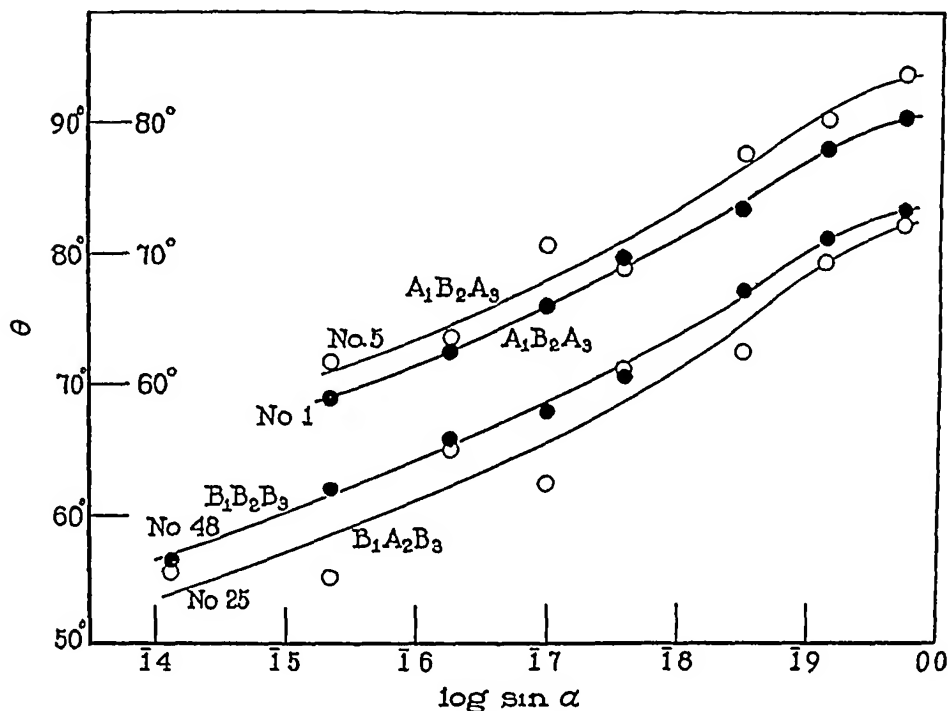
FIG 3 There occur two rather clearly defined classes of young rats in the backcross $F_1 \times A$ as regards the type of scatter of mean θ as a function of sin α, this is shown in typical example both for rats which do not orient at $\alpha = 15°$ and for those which do   The subsequent analysis indicates the basis upon which the particular individuals for which data are here plotted are assigned the respective genetic compositions indicated, individuals Nos 5 and 25 illustrate the type of scatter which is labelled R (rough), individuals Nos 1 and 48 the much closer adherence to a smooth curve which is labelled type S (smooth)

to be characteristic of the B type lower portion of the θ—α curve, a slope $\alpha = 20°$ to be characteristic of the A type   We would expect the appearance of these two classes with equal frequencies, and without intermediates, if B_1 and A_1 are inherited alternatively and if no impor- tant "modifiers" are operative   It is to be emphasized that it is not

intended to imply that $\alpha = 15°$ and $\alpha = 20°$ are peculiarly "absolute" threshold slopes both in A and in B pure lines of rats the threshold slope varies slightly, and is difficult to determine within $\pm 2°$, but all A rats orient at $\alpha = 20°$, and do not at $15°$, and all B rats (and $F_{1(B \times A)}$ and $F_1 \times B$) orient at $15°$, it is probable that in the cross bred progeny small modifications of statistically recognizable threshold slopes could be found if sought for, but we are concerned only with the large difference which is unmistakable   Of the forty-one $\Gamma_1 \times A$ individuals tested, twenty-one showed definite orientation at $\alpha = 15°$, twenty only at $\alpha = 20°$ On the basis we have thus far indicated, this corresponds quantitatively with the expectation that there is a clean cut segregation of B_1 and A_1 factors in the backcross progeny Of the twenty-one orienting at $\alpha = 15°$, ten are female and eleven male Of the twenty orienting at $20°$, but not at $15°$, six are female, fourteen male

The case is in reality more complex   We have also to deal with the fact that in $F_{1(A \times B)}$ there was evident a broadening of θ—α plot in the low α zone corresponding to the activity of excitation units of group 1 An effect of this sort must be recognized in certain of the individuals in the present backcross population   It is illustrated in Fig 3, and is recognized as an enhanced lateral scatter of the determinations of mean θ The individual curves exhibiting this scatter have been labelled "rough," R, those not exhibiting it are by contrast "smooth," S Of the forty-one tested individuals, twenty two and possibly two others, gave curves plainly showing the R type of spread (Fig 3), seventeen definitely showed the S character (these are indicated in Table II) The occurrence of these two types, S and R, in relation to threshold α and to sex, is as follows

Type of curve	R		S	
Threshold α	15	20	15	20
No of cases	12	12	9	8
♂♂	6	6	5	7
♀♀	6	6	4	1

It is to be noted that any "accidental" irregularities in the determination of mean θ's would lead to an over-estimate of the R group

The R effect was attributed (Crozier and Pincus, 1931–32 b) to a developmental disharmony, in the $F_1 \times B$ population it was not de

tectable   On this basis about half the B_1 type rats in the $F_1 \times A$ generation should show the effect, the others not

The frequency distribution of mean θ for $F_1 \times A$ rats at $\alpha = 15°$, consequently of those assumed to be of B_1 type, is sharply monomodal (Fig 4), the peak coinciding with the θ already known for $F_{1(A \times B)}$ and
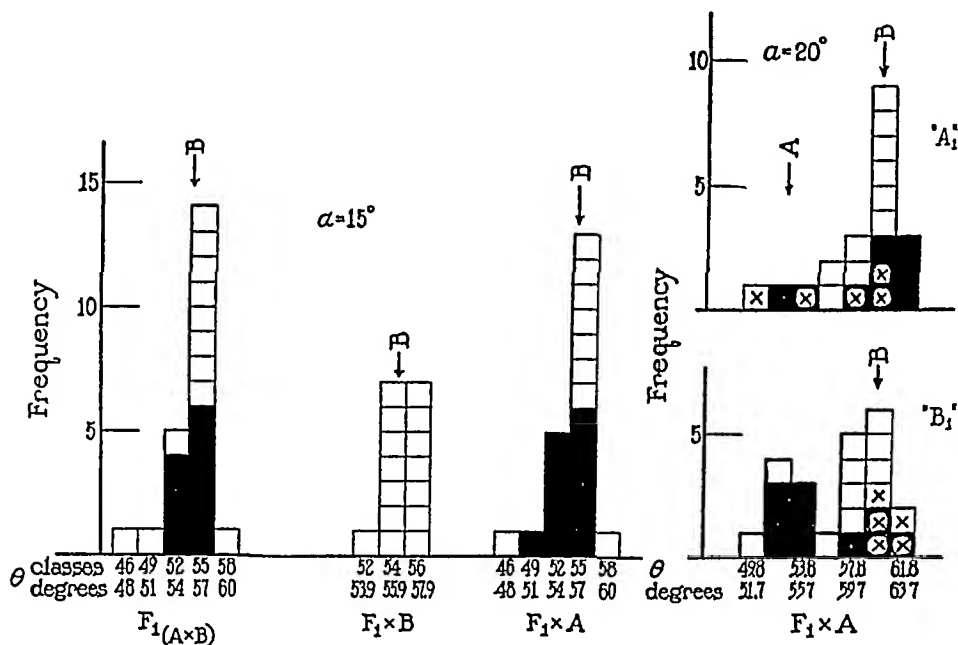
FIG 4   Frequency distribution of mean θ for various types of individuals produced in matings of strains A and B—F_1, $F_1 \times B$, and $F_1 \times A$ Of the latter there are two categories, those which orient at $\alpha = 15°$, and which show a modal θ agreeing essentially with that obtained with F_1 and with pure B, of the latter, orienting at threshold $\alpha = 20°$, but not at 15°, the majority show a mean θ agreeing with that already known for race B Of the individuals orienting at 15°, something more than one-half show a mean θ at $\alpha = 20°$ which corresponds with that found for race B, the remainder a modal mean θ corresponding to that known for race A The reasons for this are discussed in the text, where the symbols are explained

for $F_1 \times B$ (Crozier and Pincus, 1931–32 b)   The S and R types do not differ in this, in Fig 4 the R type is shown in solid squares, the S open   The distribution of P E $_\theta$ is similarly like that for F_1 and for $F_1 \times B$, though for $F_1 \times A$ its mode is a little higher, like that for $F_{1(A \times B)}$ with B ♀ parent

At $\alpha = 20°$ the B_1 type rats—those already seen to orient at $\alpha = 15°$, —show two modal θ s, one is rather close to that typical of pure A, the other is equivalent to that for pure B Closer inspection indicates that the individuals as subsequently classified on the basis of the shapes of their orientation curves exhibit a significant correlation with these findings   The young rats thus presumed to have the phenotypic composition $B_1A_2B_3$ have a lower mean θ at $\alpha = 20°$ than do the others, the presence of an A_2 group (homozygous) lowers the B_1 graph, whereas with A_1 individuals, all those not $A_1A_2A_3$ in composition show a B
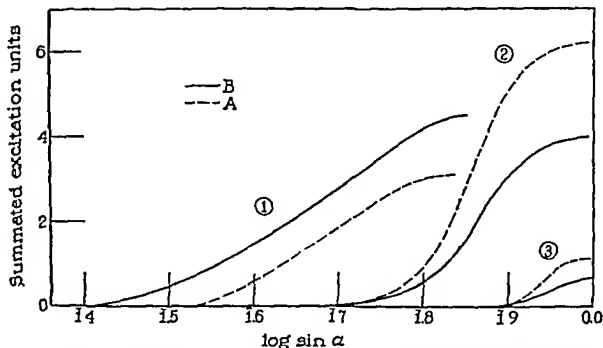
FIG 5   Integrals of populations of receptor units in terms of thresholds, receptor groups 1, 2, and 3 for races A and B See text

modal θ at $\alpha = 20°$, a B_2 or B_3 group (or both) serving to raise the A_1 graph   In the right hand histograms of Fig 4, the solid squares are from R type curves, the $\times$ indicates $B_1B B_3$ Consequently, the classification of the $B_1 \times A$ population must be based primarily upon the shapes of the individual θ—α curves

IV

The simplest method of dealing with the degree of correspondence between expectation and the observed θ—α curves would be to construct theoretically the curves for the classes of segregates to be looked for, on the assumption that the several groups of excitation units are

Mean orientation angles for progeny obtained in the cross $F_1 \times A$, the individuals are grouped according to the slopes of their θ — log sin α curves into eight categories, as discussed in the text   In each class the individual mean θ's have been averaged   The P E's of the individual mean θ's are given, $n = 20$ in each case   At each slope of surface, within each class of rats, these P E's have been averaged   The subscript number attached to the identifying number of each individual indicates the litter of which it was a member   The symbols S and R are referred to in the text

II

$B_1B_2A_3$

No		α, degrees							
		15	20	25	30	35	45	55	70
22_3	♀ S	46 5±2 57	49 8±2 21	57 6±1 90	61 3±1 55	67 7±1 24	72 6±1 04	76 6±1 08	79 4±1 01
29_6	♀ S	54 4±2 06	60 0±2 39	64 7±1 54	66 0±1 42	73 0±1 53	75 2±1 02	79 6±0 970	82 4±0 669
46_7	♂ R	54 7±2 42	58 9±2 10	57 1±2 92	67 3±1 79	64 7±1 63	70 3±1 26	74 8±1 59	79 7±1 20
$*50_8$	♀ S	56 0±2 08	60 8±2 10	64 7±1 83	69 1±1 66	75 2±1 32	76 6±1 66	78 3±1 20	82 0±0 776
53_9	♂ S	55 2±2 30	58 5±3 12	62 7±1 97	63 4±2 00	68 8±1 87	72 8±1 42	78 4±1 08	82 0±0 862
Mean θ		53 4±0 922	57 6±1 06	61 4±0 844	65 4±0 731	69 9±0 682	73 5±0 558	77 5±0 488	81 1±0 362
Mean P E θ		2 28	2 38	2 03	1 68	1 52	1 26	1 18	0 904

* Nursed by F_1 No 7

$B_1B_2B_3$

No		α, degrees							
		15	20	25	30	35	45	55	70
6_2	♀ R	54 8±2 00	63 0±1 91	64 1±2 09	63 8±1 98	71 5±1 85	77 2±1 30	81 7±1 07	82 4±1 09
21_3	♀ R	54 9±1 84	60 9±2 30	60 6±2 38	66 9±1 39	71 7±1 08	74 8±1 48	81 1±0 83	82 2±0 919
23_3	♂ S	56 4±2 40	61 0±2 29	65 5±1 36	67 6±1 78	73 0±1 16	77 8±0 959	82 4±0 873	83 6±0 776
33_6	♀ R	56 2±1 93	60 4±2 42	61 4±1 88	66 4±1 48	74 3±1 29	75 4±0 912	82 5±0 862	83 5±0 717
48_8	♀ S	56 5±2 72	62 0±2 12	65 8±1 70	67 9±1 97	70 6±1 58	77 1±1 26	81 1±1 07	83 2±1 02
Mean θ		55 76±0 922	61 46±0 911	63 44±0 776	66 52±0 710	72 55±0 574	76 46±0 488	81 76±0 383	82 98±0 373
Mean P E θ		2 18	2 21	1 88	1 72	1 39	1 18	0 940	0 906

B_1A_3

α degrees

No.		15	20	25	30	35	45	55	70
27₄	♂S	55 4±2 34	59 0±1 67	62 0±2 36	62 7±1 75	69 2±1 53	75 6±1 20	78 5±1 09	78 2±1 0₃
32₄	♀S	54 7±2 19	58 4±2 53	60 0±2 19	62 7±1 82	71 7±1 14	77 6±0 94₁	79 0±1 26	80 6±1 06
34₄	♂S	55 0±2 65	59 5±1 59	63 9±2 15	64 1±1 92	69 0±1 99	78 5±0 61₁	79 8±1 05	80 5±1 16
38r	♂S	58 1±1 82	61 0±2 49	65 2±1 68	69 9±1 79	69 0±1 77	78 0±1 05	82 2±1 89₂	81 3±0 75₆
40r	♀R	53 8±2 25	55 4±2 33	57 1±1 67	60 6±2 16	67 7±1 72	72 2±1 28	77 9±1 36	79 1±1 12
52₈	♀R	54 9±2 20	52 3±2 12	60 2±2 23	63 1±1 97	68 7±2 17	75 0±1 29	79 9±1 04	80 8±1 02
Mean θ		55 0±0 694	57 6±0 656	61 4±0 634	64 2±0 589	69 2±0 632	76 2±0 329	79 5±0 349	80 8±0 318
Mean P E θ		2 24	2 12	2 05	1 90	1 72	1 06	1 11	1 03

B_2A_3

α degrees

No		15	20	25	30	35	45	55	70
7₁	♂R	53 1±2 31	53 4±1 82	58 4±2 26	63 9±1 51	65 0±1 44	73 5±0 92₁	76 7±1 12	76 3±1 09
9₁	♂R	51 9±3 34	53 8±2 35	61 1±2 09	58 5±3 00	67 3±2 34	73 3±1 33	78 6±1 10	80 3±0 882
25₁	♂R	55 6±1 81	55 2±2 66	65 1±2 34	62 5±2 34	71 3±1 42	72 6±0 959	79 4±1 04	82 3±0 678
30₈	♀S	51 4±2 81	52 1±2 23	57 4±2 23	62 3±1 24	67 1±1 57	74 8±1 13	76 4±1 34	81 2±0 902
35₈	♂S	55 2±2 54	56 6±2 50	63 5±2 09	63 3±1 99	71 9±1 32	76 7±1 24	78 3±1 10	81 7±0 727
55₅	♂R	53 3±4 15	52 7±3 15	60 9±1 88	63 2±2 23	72 2±1 54	76 8±1 65	83 1±0 805	85 9±0 ₃33
Mean θ		52 9±0 773	54 0±0 764	61 1±0 659	62 3±0 635	69 1±0 497	74 6±0 373	78 8±0 336	81 3±0 258
Mean P E θ		2 49	2 45	2 13	2 05	1 60₄	1 20₄	1 08₃	0 801₆

TABLE II—*Concluded*

$A_1A_2A_3$

No		20	25	30	35	45	55	70
					α, *degrees*			
3_1	♂R	58 9±2 96	57 3±2 04	61 6±2 34	68 4±1 36	70 6±1 92	76 6±1 77	80 3±1 27
8_2	♂S	61 0±2 06	60 1±1 64	66 8±1 87	69 7±1 24	75 5±1 58	78 1±1 28	80 9±1 25
31_6	♂S	50 3±1 66	56 9±2 04	63 9±2 24	69 3±1 15	73 4±1 05	76 5±0 97	80 1±0 87
36_7	♀R	54 9±2 66	58 1±2 24	61 4±1 91	64 8±1 91	69 3±1 22	76 5±0 57	80 1±0 70
54_9	♂R	60 1±1 80	56 7±2 30	63 1±2 12	71 3±1 48	77 4±1 19	83 5±0 57	83 6±0 69
Mean θ		57 1±1 00	58 8±0 853	63 4±0 866	68 8±0 593	73 2±0 612	78 2±0 426	81 0±0 414
Mean P E θ		2 23	2 05	2 10	1 43	1 39	1 02	0 960

$A_1A_2B_3$

No		20	25	30	35	45	55	70
					α, *degrees*			
2_1	♀S	57 1±2 42	62 7±1 72	63 6±2 54	69 2±1 54	77 4±0 843	78 1±0 959	80 5±0 912
4_1	♂R	61 1±1 71	66 1±1 27	62 4±1 78	66 5±1 68	74 6±1 13	81 3±0 853	81 2±1 14
37_7	♂R	63 2±1 88	65 5±1 57	66 6±1 74	68 6±1 75	77 1±1 11	80 2±1 01	82 0±0 991
*51_8	♀S	59 3±2 65	62 5±2 70	68 6±1 60	72 5±1 12	77 6±1 22	82 2±0 805	81 6±0 873
Mean θ		60 2±1 07	64 2±0 898	65 3±0 949	69 2±0 752	76 7±0 533	80 5±0 447	81 3±0 485
Mean P E θ		2 14	1 82	1 92	1 52	1 08	0 91	0 98

* Nursed by F_1 No 7

A₁B₂

No		0	25	30	35 σ degrees	45	55	70
20₂	♂S	60 7±2 47	64 0±2 21	65 5±1 52	72 6±1 41	¦9 1±0 640	82 1±0 668	83 3±0 853
26₄	♀S	60 4±2 34	62 9±1 74	65 7±1 50	70 6±0 582	74 6±1 48	79 5±1 23	81 2±0 804
28₈	♂S	61 4±2 70	64 6±2 29	66 2±2 02	72 1±0 970	77 8±1 13	81 4±1 03	82 8±0 766
56₉	♂S	55 9±2 16	61 0±1 45	65 5±1 98	74 9±1 33	76 0±1 21	82 1±0 902	82 1±0 736
Mean θ		59 6±1 16	63 1±0 960	65 8±0 86	72 3±0 632	76 9±0 553	81 3±0 474	82 4±0 416
Mean P E θ		2 42	1 92	1 75	1 07	1 11	0 95	0 79

A₁B₃

No		20	25	30	35 σ degrees	45	55	70
1₁	♂S	59 0±1 77	62 6±1 33	66 0±1 45	69 7±1 32	73 4±0 931	77 9±0 785	80 2±1 30
5₁	♀R	61 8±2 33	63 6±1 91	70 7±1 29	68 9±2 05	77 6±0 941	80 1±1 16	83 6±0 717
24₄	♀R	52 7±1 73	62 0±2 06	62 3±2 04	68 5±1 56	73 6±1 17	78 2±1 19	79 4±1 10
39₇	♂R	60 2±1 45	65 5±1 83	66 5±1 14	70 3±1 11	73 1±1 59	79 0±0 981	79 7±0 892
47₈	♂R	62 5±2 40	63 4±2 16	71 2±1 45	71 9±1 14	73 1±1 72	79 7±0 737	79 1±0 843
49₉	♀R	61 1±2 14	61 4±1 75	61 8±1 88	70 3±1 88	71 0±1 43	78 8±1 01	81 8±0 862
Mean θ		59 6±0 610	63 1±0 569	66 4±0 478	69 9±0 468	73 6±0 402	79 0±0 303	82 0±0 295
Mean P E θ		1 97	1 84	1 54	1 51	1 29	0 980	0 951

transmitted independently   The three components of the curve
$\Delta\theta/\Delta$ log sin α *vs* sin α for each of the grandparental strains (Crozier
and Pincus, 1929–30 *b*, etc) might be integrated separately and the
various possible phenotypic combinations constructed by suitable addi-
tion of B_1 with A_2, A_1 with B_2, and so on, thus producing θ—α curves
for the several types of individuals expected to exhibit the correspond-
ing assortments of genetic units   The integral forms of the component
receptor groups are given in Fig 5   Six such combinations are in
prospect, in addition to the grandparental types   Two obstacles,
however, prevent the precise use of such a method, even were the
available data much more extensive   We do not know precisely
enough the exact threshold slopes of surface   This means that even
with the pure lines A and B there is an uncertainty about the absolute
values of the ordinate scales (θ) in terms of a common standard, a
common standard must be employed if B_1 and A_2 are to be added
For example, B_1 as given by its differential plot (*cf* Crozier and Pincus,
1929–30 *b*) corresponds to 14 4 θ units, *in the B stock* for which θ
increases 29° above the threshold response $\theta = 55°+$, A_1, in corre-
sponding units $= 7$ 94 above a threshold θ of 52 8° for the A race
But in the backcross individuals we apparently have some A_1 type
with a B_1 type threshold θ, which gives the second obstacle to the sug-
gested treatment   These integrations have been carried through, how-
ever, for their suggestive value, various assumptions regarding the
nature of the measurable threshold response have been tested, but need
not be given here since they do not necessarily add anything to the
classification later made by rougher means, the curves in Fig 5 are
useful as indicating merely the *kinds* of θ—α curves to be expected
We have already commented upon the fact that in the data from cross-
bred populations slight shifts are apparent in the modes of the receptor
groups   This may be taken to suggest that certain of the subsidiary
modifications of the slopes of the θ—log sin α curve could be due to
changes in the relation of frequency of stepping to sin α, the frequency
of leg movements (speed of progression) is involved in the logarithmic
differentiation of θ by sin α from which the frequency distribution of
receptor thresholds is deduced (Crozier and Pincus, 1929–30 *a*)   It
may also be taken to indicate that even in the case of rats from inbred
lines (A, B, etc) a more elaborate determination of the θ—log sin α

curve, employing a larger number of slopes of surface, might reveal detectable discontinuities of small magnitude

Confining attention to the slopes of the θ—α curves, and particularly to the regions where the relatively uncomplicated effects of groups *1*, *2*, and *3* might be looked for, the classification outlined in Table II is arrived at

It need not be emphasized that there are various pitfalls   We can not as yet be entirely clear as to the effect upon an A_1A_2 type curve
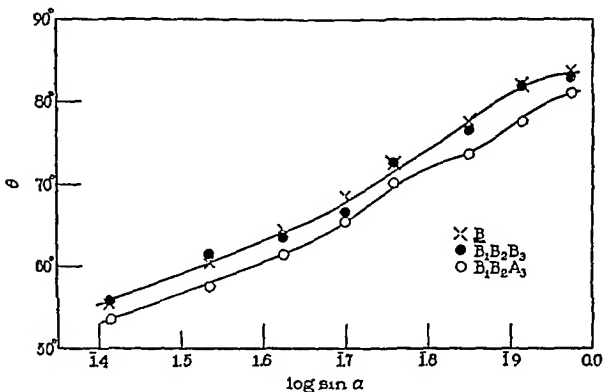
FIG 6 θ as a function of log sin α for pure line B is essentially reproduced in the behavior of individuals extracted from the backcross $F_1 \times A$ taken to have the composition $B_1B_2B_3$ The curve is also shown for individuals assigned the composition $B_1B_2A_3$ this curve is lower than the foregoing (see text)

when it has a B_1 type threshold α But with reference to their shapes, the individual curves do, however, fall into certain definite categories It is simplest to begin with those curves which appear to be of B type throughout, since experience with F_1 progeny of $A \times B$ has already demonstrated certain sources of confusion which effectively disappear when these are backcrossed to the B stock   Such individuals are found in Nos 6, 21, 23, 33, and 48   For these the variation data (Figs 8, 9, and 10) also show internal concordance   For those indi

viduals taken to be $B_1B_2B_3$ the *percentage modifiable variation* is 65 5 per cent, identical with that for the phenotypically $B_1B_2B_3$ type obtained by crossing F_1 with the B line, V N $_{\log \sin \alpha}$ and V N $_{\sin \alpha}$ are a little less, as is also the total mean variation of θ, the latter is about as in the B stock   This is entirely in agreement with what we have already had reason to expect (*cf* Crozier and Pincus, 1931–32 *e*) on the basis of evidence published before the present data were reduced
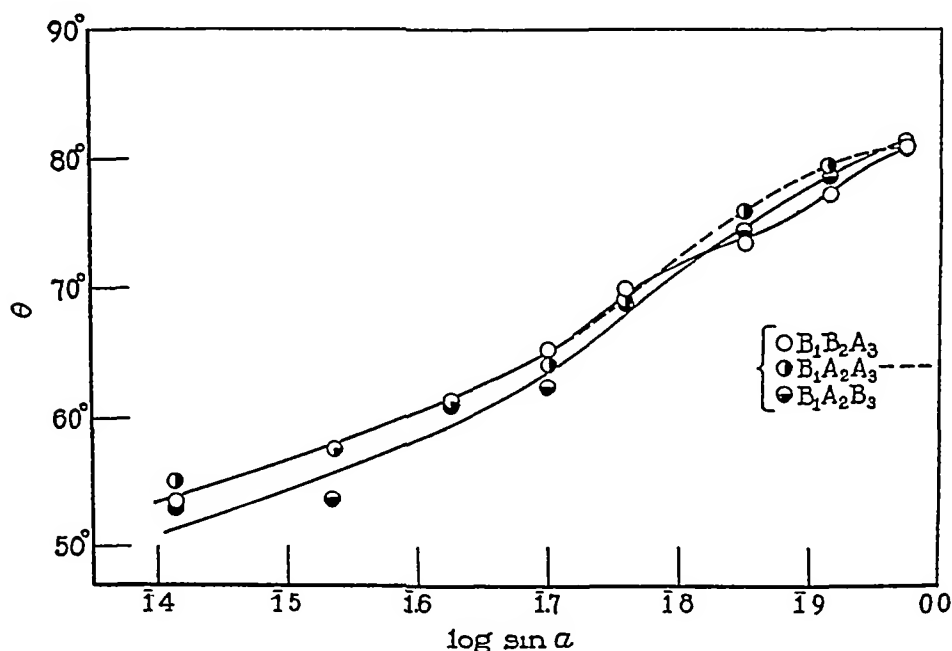
FIG 7   Mean orientation angles (θ) for groups of individuals assigned the compositions $B_1B_2A_3$, $B_1A_2A_3$, $B_1A_2B_3$

As seen in Fig 6, the $B_1B_2B_3$ group gives mean θ's agreeing very precisely with data from a group of great-grandparent pure B's, the plotted points are of about the same statistical weight in the two sets compared   Whereas the $B_1B_2A_3$ group gives a curve definitely lower at all points, and of different shape (Fig 6), $B_1A_2A_3$ and $B_1A_2B_3$ mean curves differ as expected (Fig 7), the decided changes of slope occur in the regions where the separate curves in Fig 5 require if the total effect is an additive one   The agreement of $B_1B_2B_3$ with its B progenitors in the matter of variation is striking   In Fig 8 there have been plotted data on the variation of θ as a function of $\sin \alpha$ for four

assemblages of young rats independently assigned the phenotypic com
position $B_1B_2B_3$ pure B's (great grandparents), $\Gamma_{1(A \times B)}$, $F_1 \times B$,
and the present lot (B_1B B_3 in Table II)   The data are given as *mean*
P E $_\theta$ computed from P E $_\theta$'s for $n = 20$ in each case   It is obvious
that the capacity to exhibit variation of θ is identical in the four
groups   This kind of invariance is also evident in the case of the
$A_1A_2A_3$ lot and its homologs (*cf* Fig 12, etc), and is all the more strik
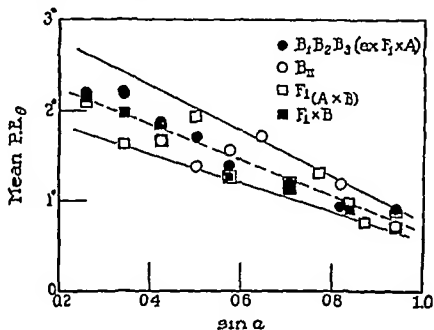
FIG 8   The variation of mean θ as a function of sin α for four groups of indi
viduals recognized as of the phenotypic composition B_1B B_3 solid circles, ex
tracted $B_1B_2B_3$ in the present experiment open circles, Series B II (Crozier and
Pincus 1931-32 a) open squares $F_{1(A \times B)}$ (Crozier and Pincus, 1931-32 d) solid
squares, $F_1 \times B$ (Crozier and Pincus, 1931-32d)   The ordinates are *mean*
probable errors of mean θ s for twenty observations on each individual at each
slope of surface the numbers of individuals differ in the several sets   It is ap
parent that the *variability* of θ is identical in these phenotypically similar groups
of individuals

ing when the variability of θ is examined in the case of the other group
ings, where definite discontinuities appear   Fig 9 contains the varia
tion data for the several groups of B_1 type rats, $B_1B_2B_3$, $B_1B_2A_3$,
$B_1A_2B_3$, $B_1A_2A_3$ The individuals are separately distinguished   It is
apparent that on the basis of variation of θ alone, the recognition of
the four sorts of B_1 type rats would be necessary and that the result of
this segregation agrees in a very striking manner with that already
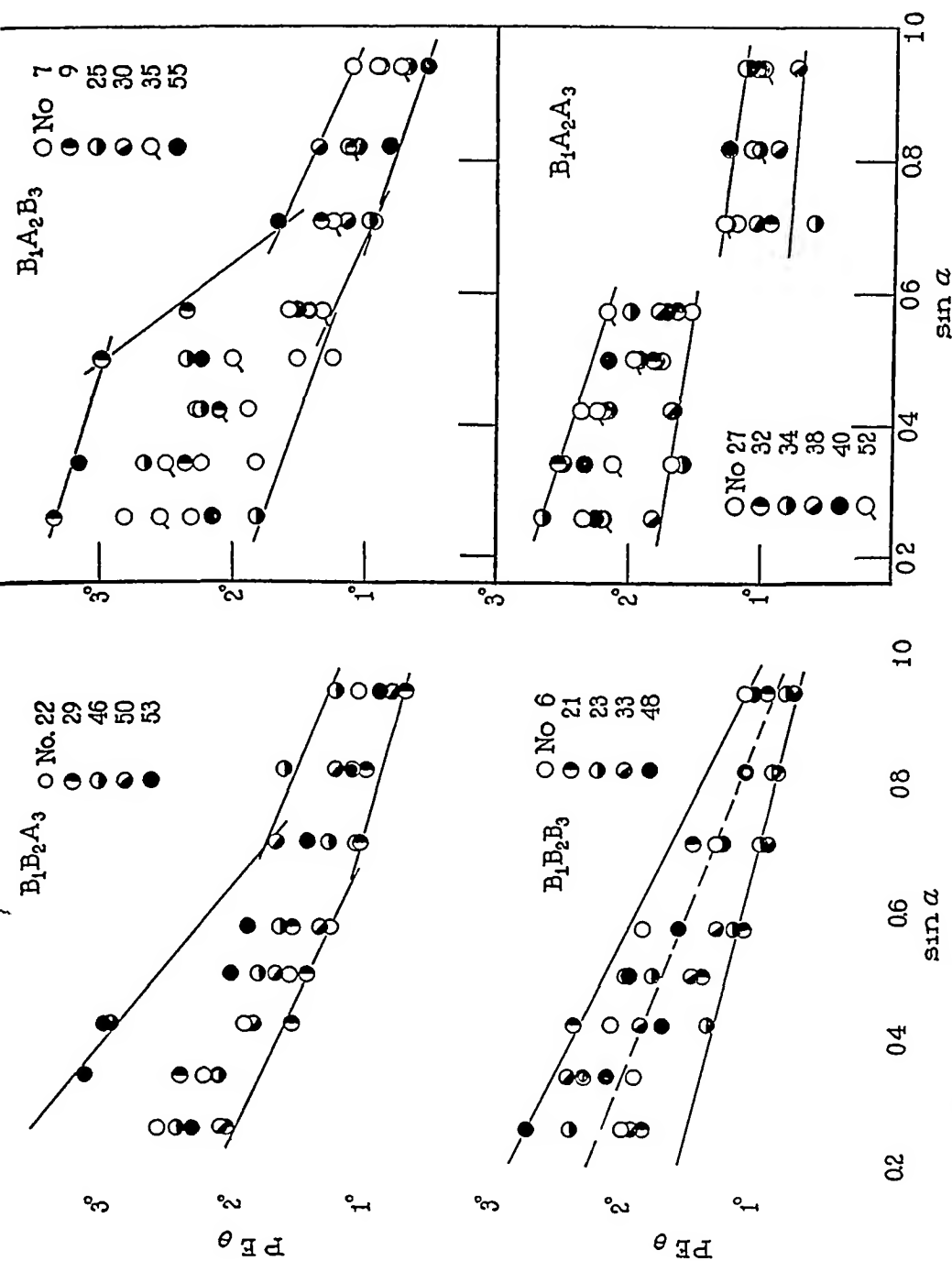
FIG 9 P E of mean θ for various individual young rats grouped on the basis of their θ-log sin α curves into four phenotypic categories   Each P E θ is computed as the P E of the mean of twenty observations   It is apparent that there is essential coherence among the variabilities of the several individuals in each class, and that discontinuities in the succession of B and A receptor groups are clearly reflected in the variation of θ
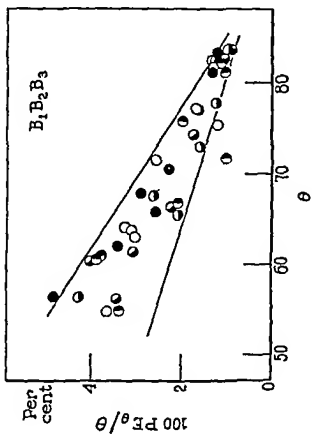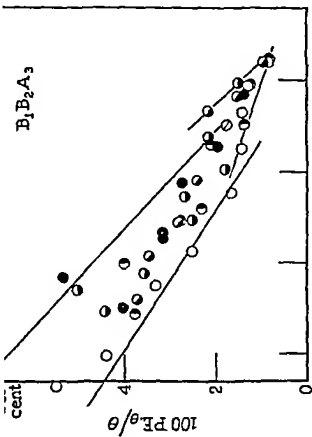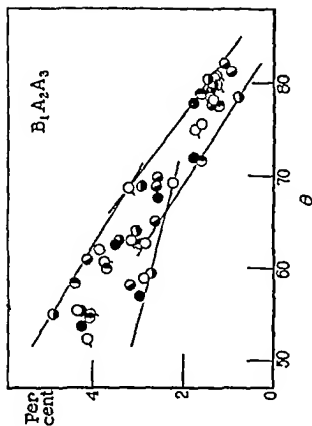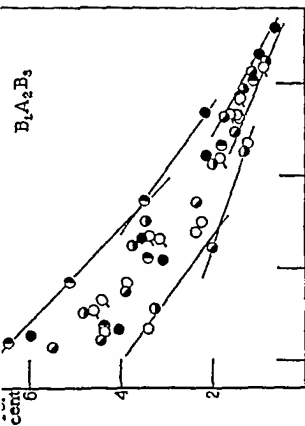
given in Figs 6 and 7   The *relative* variation of θ (Fig 10) shows cor-
responding discontinuities

The peculiar discontinuities in Fig 9 have not been encountered in
pure stocks A, B, K, or P, nor in $F_{1(A \times B)}$, failure of material has pre-
vented our re-examination of this point for R *rattus* (Crozier and Pin-
cus, 1926–27 b)   It would be of more than passing interest to learn
why P E $_\theta$ is smoothly related to sin α in the pure stocks, and if long
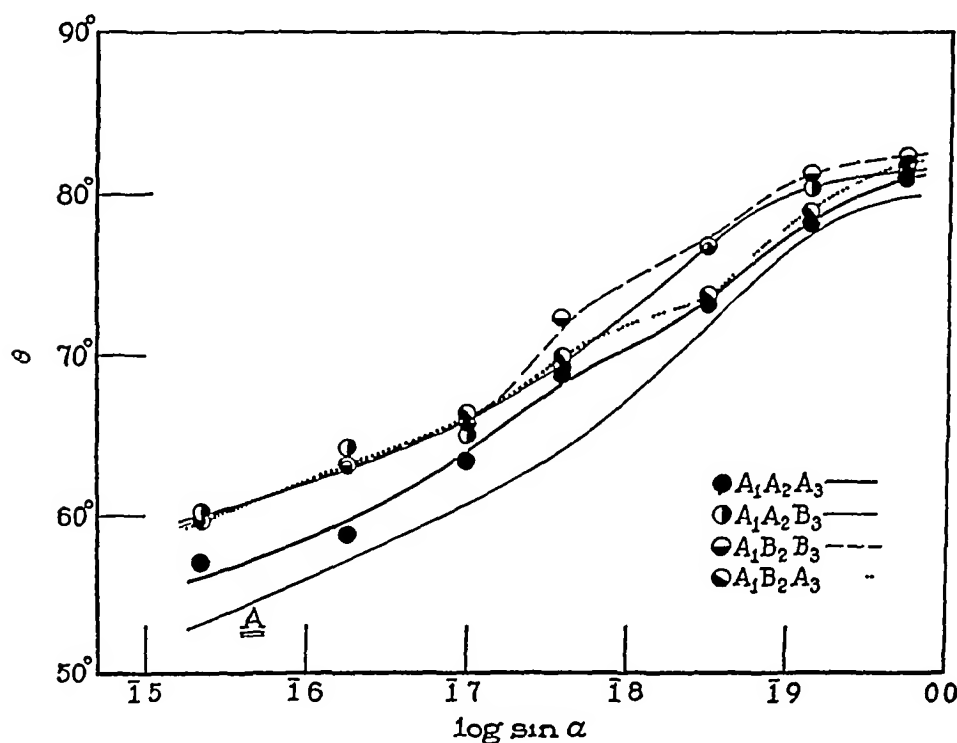
FIG 11   Mean orientation angles (θ) for four groups of young rats assigned the
phenotypic compositions indicated, together with the curve for the A line great-
grandparents

inbreeding of the heterozygotes (e g , $B_1A_2A_3$) would modify the effect
seen in Fig 9   The possible rôle of influences affecting the frequency
of stepping we have already commented upon

The four classes of A_1 type rats grouped by use of the same criteria
as those used for the B_1 rats give mean θ's plotted in Fig 11   In fitting
curves to these they have been made to pass as smoothly as possible
and in such a manner that the departure of any point from its curve is

less than the probable error of its mean   Again the form of each
curve is that which would be expected from the association of receptor
groups indicated   All four curves are above that for pure A stock,
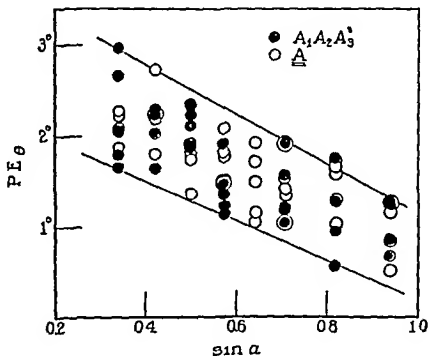
FIG 12 The P E of mean θ twenty observations for each point, for individuals of A (Series IV) and for individuals produced in the backcross $F_1 \times A$ to which have been assigned the phenotypic composition $A_1A_2A_3$
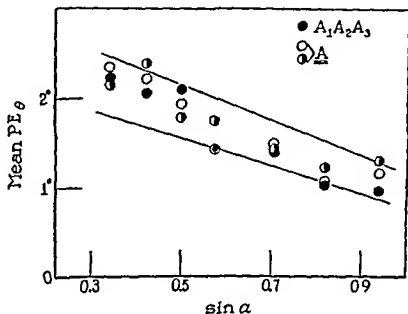
FIG 13 The *mean* P.E θ (from samples of twenty observations) for young rats of line 4 and for $F_1 \times A$ extracted individuals assigned the phenotypic composition $A_1A_2A_3$
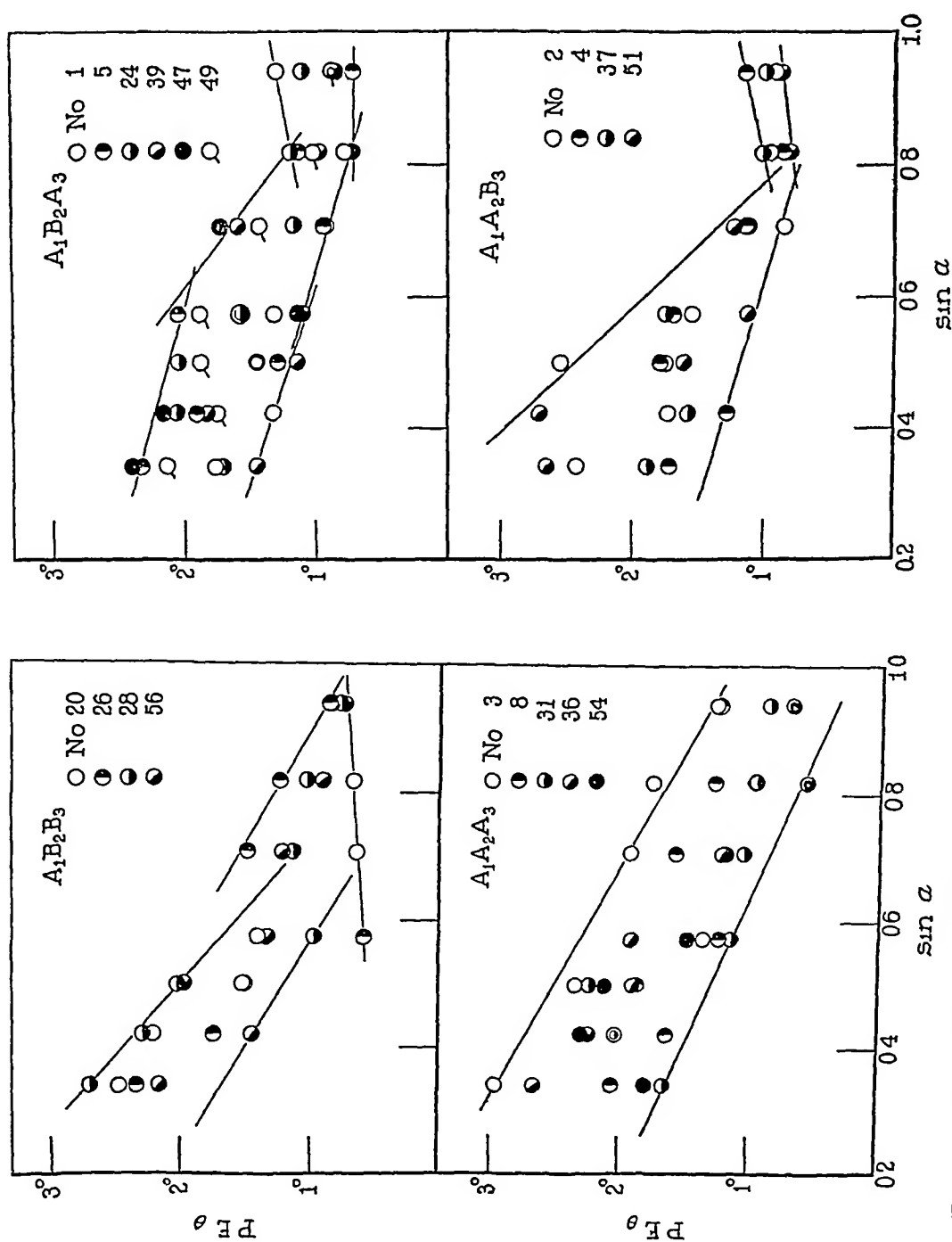
Fig 14 P E of mean θ for individuals allocated to four phenotypic categories As in the case of B₁ individuals (Fig 9), the discontinuities in curvature of the θ-log sin α plot find corresponding representation in the behavior of P E₀
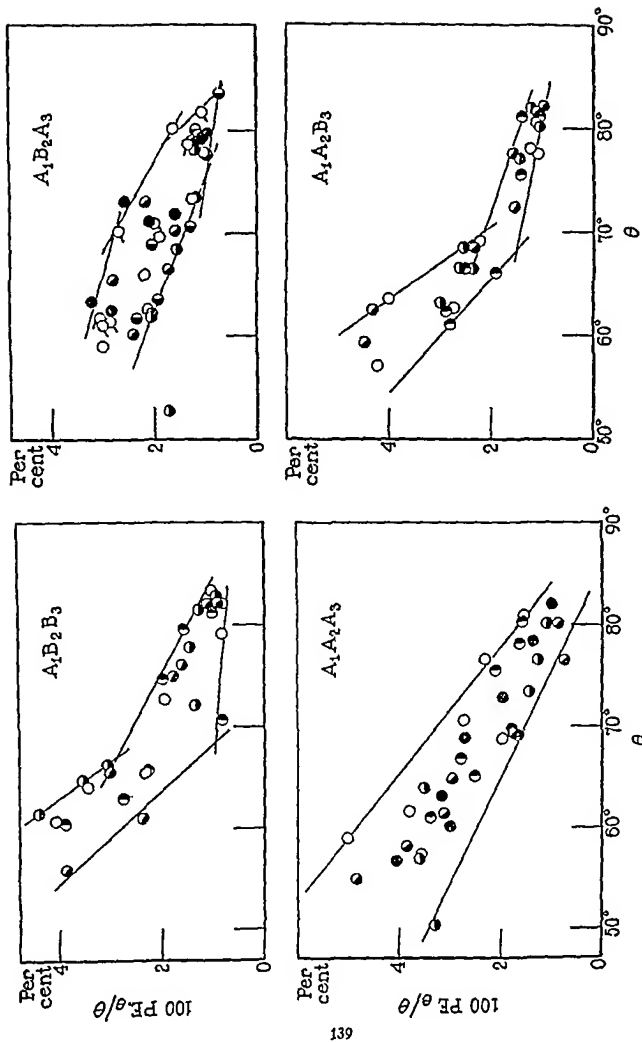
FIG 15 The peculiarities of P E$_\theta$ as a function of sin α (Fig 14) are reflected in the behavior of the relative variation of θ

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this presumably is due to the action of accessory influences introduced by the B stock, of the type to which reference was made in the introductory account   The argument here is a duplication of that already outlined for the interpretation of the data in the B_1 assemblage of rats The variation of θ for $A_1A_2A_3$ rats agrees quantitatively with that for pure A, as shown in Figs 12 and 13, the *modifiable variation* is less (60 per cent) than for $B_1B_2B_3$, for the other three groups discontinuities appear in the relation of P E $_\theta$ to sin α which are of precisely the same character as those exhibited in the four B_1 groups (Figs 14 and 15, *cf* Figs 9 and 10)   The coherence of the complex system of indications provided by these figures is strong evidence that their suggestions are significant   The discontinuities apparent in the graphs of variation of θ as a function of sin α, and of θ, are of course consistent with the general idea that the *total* variation of performance (in an inbred line) is determined by the total number of excitation units available   Discontinuity of variability appears in the region of transition from one receptor group (Fig 5) because the marginal receptors in use determine the rate of change of variation of θ

<center>V</center>

We are thus in a position to conclude that a consistent picture of the inheritance of geotropic performance in crosses of rats of lines A and B is given by the theory that the three recognizable receptor groups concerned in each race are inherited independently, and allelomorphically as concerns the members of homologous pairs in the two races The shapes of the curves produced by the backcross progeny are understandable in this way, as well as the thresholds for oriented response and the peculiar properties of the variation of orientation   To this simple picture of the genetic basis for phenotypic segregation must be added, however, certain comparatively minor modifying effects which have been characterized as disharmony due to heterozygosis (factors introduced by the B stock) and the influence of association between B and A factors phenotypically exhibited upon the magnitude of response at threshold intensity of excitation

It is of interest to make a direct test of the validity of the grouping of individuals in the $F_1 \times A$ generation which has appeared consistent with this interpretation   To this end rat 24 ♀ (*cf* Table II) was

TABLE III

Progeny of 24 ♀ $(F_1 \times A) \times A$

No	20	25	30	35	45	55	70
				σ deg res			
				$A_1D \cdot A_3$			
1 ♀	53 0±2 28	60 1±2 12	66 3±1 99	73 3±1 22	71 2±1 29	79 0±0 912	80 3±0 941
2 ♂	56 1±2 87	62 9±2 43	67 8±1 76	70 8±1 42	75 2±1 20	78 6±1 05	79 9±1 04
3 ♂	56 3±2 02	59 8±2 15	66 0±2 06	72 4±1 02	72 6±0 989	78 7±0 922	80 5±1 15
Mean θ	55 1	60 9	66 7	72 2	73 0	78 7	80 2
Mean P E θ	2 39	2 23	1 94	1 22	1 16	0 960	1 04
				$A_1A\ A_3$			
4 ♂	53 9±2 52	58 5±2 34	62 4±2 31	66 9±2 02	71 7±1 85	77 9±0 941	79 3±0 940
5 ♀	53 9±2 45	60 9±2 02	64 2±1 66	60 6±1 81	72 8±1 63	78 9±0 883	77 3±1 07
Mean θ	53 9	59 7	63 3	66 5	72 3	78 4	78 3
Mean P E θ	2 40	2 18	1 99	1 92	1 74	0 912	1 01
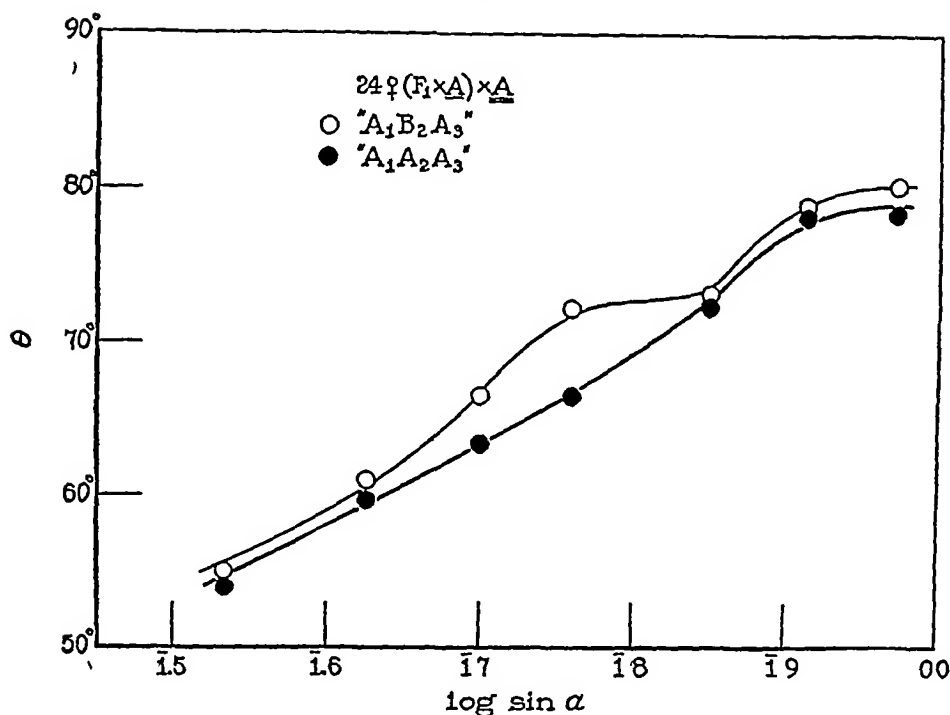
FIG 16 Curves exhibiting θ as a function of log sin α for two recognizably distinct groups in the progeny from a mating of No 24 female $(F_1 \times A) \times A$ (see text)
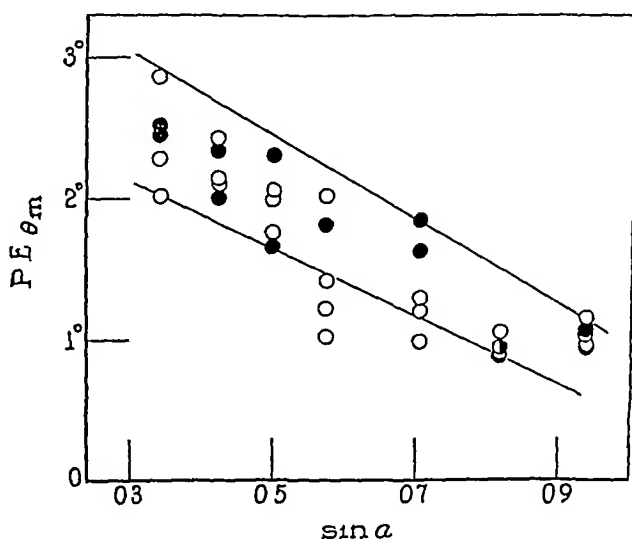
FIG 17 P E 's of mean θ for individuals produced in the mating of $(F_1 \times A)$ No 24 female with an A male, the solid circlets referring to those individuals recognized as of the type $A_1A_2A_3$, the open circlets referring to those individuals recognized as $A_1B_2A_3$ See text

raised to maturity and mated to an A line male   The five young were examined for geotropic orientation   This particular rat was chosen because she had been assigned the composition $A_1B_2A_3$, with group 2 heterozygous (since No 24 had resulted from $F_{1(A \times B)} \times A$)   The progeny were thus necessarily expected to be of two types only— $A_1A_2A_3$ and $A_1B_2A_3$ The data are given in Table III   There are clearly enough two classes of young   Mean θ's for each are plotted in Fig 16, they correspond well with the two types so labelled in Fig 11   The variation of θ shows (Fig 17) a concordant separation into two types, the $A_1B_2A_3$ group exhibiting breaks of the sort already seen in Fig 14, a larger series would permit the treatment of certain minor differences in these figures which are presumably due to the continuation of backcrossing

VI

SUMMARY

The inheritance of elements of geotropic performance in lines of rats (A and B) has been investigated by examining the orientation of young offspring produced in matings of $F_{1(A \times B)}$ with A Previous studies had shown that the three recognizable groups of receptor elements concerned in geotropic orientation in each of these lines appeared to be inherited in such a way that B groups were dominant with respect to A groups, although this was to a minor extent complicated by influences affecting the variation of orientation as well as the exact form of the curve relating orientation angle (θ) to slope of surface   In the backcross $F_1 \times A$, therefore, at least eight different types of curves were to be expected   These are in fact identifiable among the forty-one individuals carefully studied   Their classification is concordant with the behavior of the respective indices of variation of θ, for which an interpretation has been provided   The basic result is, therefore, that the three receptor groups of excitation units are inherited independently, and alternatively as regards the members of a homologous pair, and that rather simple dominance relations obtain between homologous groups from the two races, namely that a B effect is dominant over the homologous A effect   This interpretation has been tested in various ways, and is in principle completely consistent with the results of a similar experiment involving races A and K

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STUDIES ON CELL METABOLISM AND CELL DIVISION

I On the Relation between Molecular Structures, Chemical Properties, and Biological Activities of the Nitrophenols*

By G H A CLOWES AND M E KRAHL

(From the Lilly Research Laboratories, Marine Biological Laboratory Woods Hole)

INTRODUCTION

In the course of an investigation (5 6 13) in which the eggs of the

extremely small concentrations of 4,6-dinitro *o* cresol induce a division block, third, that the block to division appears to occur most readily at the prophase of mitosis, and fourth, that the block to division is fully reversible over a wide range of concentrations

That the dinitrophenols exert an adverse effect on growing organisms at relatively high concentrations is by no means a new observation since the toxicity and insecticidal properties of these substances have

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been recognized for some years (9, 19)   However, the fact that a reversible block to cell division commences at the point where respiration is stimulated to a maximum suggests that an intensive and systematic study of the means whereby this respiratory stimulus is effected should lead not only to a more comprehensive understanding of the factors underlying the metabolism of normal and pathological cells and possible means of controlling the therapeutic application of metabolic stimulants, but might also throw light on the mechanism of cell division and afford some indication as to how cell division might be controlled

In a further series of experiments, conducted on yeast, 4,6-dinitro-*o*-cresol was found to markedly decrease the time of reduction of cytochrome (14) and to be capable of producing a stimulating effect on anaerobic systems fully equal to that exerted on aerobic systems (15) These findings make it clear that the dinitrophenols do not, like methylene blue and other reversible dyes, function in the cell as oxidation-reduction systems which supplement the normal oxygen activating enzymes   A similar conclusion has been reached by DeMeio and Barron (7)

The inability of Greville and Stern (10) to demonstrate that dinitrophenols are capable of reversible oxidation-reduction in the range of potentials characteristic of the living cell raises a doubt as to whether the oxidation-reduction of nitrophenols plays any rôle in the metabolic and division effects produced by these reagents

The experiments to be presented in the present and succeeding papers are concerned with the effects exerted by a series of nitro and other derivatives of phenols on the respiration and cell division of *Arbacia* and other marine eggs   To differentiate between the effects on resting and dividing cells, a few experiments were carried out on unfertilized eggs

An effort has been made to determine what chemical groupings and what variation in configuration of given chemical groupings are required for the production of a substantial respiratory stimulation and a reversible block to division in fertilized *Arbacia* eggs, and, in particular, to determine whether either the hydroxyl or nitro groups, or both, are indispensable   The three isomeric mononitrophenols, and several dinitrophenols were employed   Then substituent groups

derived from the nitro group by partial and complete reduction were used in place of the nitro group, keeping the phenol nucleus intact Following this, the nitrobenzene nucleus was retained while other substituents were used in place of the hydroxyl Finally, for purposes of comparison, a series of oxidation reduction indicators was employed

EXPERIMENTAL PROCEDURE

Measurements of oxygen consumption were made with simple Warburg manometers and flasks by the direct method, using essentially the technique described by Dixon (8) For determination of respiratory quotient, CO_2 was first absorbed in saturated Ba $(OH)_2$ and then liberated by 2.5 N HCl The respiratory CO_2 was calculated as the difference between the total amount present at the end of an experiment, and the initial amount as determined by liberating CO_2 from a sample of egg suspension at the beginning of the experimental period The shaking rate was sixty five complete cycles per minute at an amplitude of 7 cm

The eggs were obtained and handled in the usual manner Fertilization was performed before the egg suspension was transferred to the Warburg flasks An egg suspension of 2 per cent by volume in sea water was used If the volume of one egg is taken to be 212 000 μ^3 (12), a 2 per cent volume corresponds to approximately 90 000 to 100 000 eggs per cc The egg volume was determined by the hematocrit method (20), using a centrifugal force of approximately twenty six hundred times gravity for 10 minutes Repeated comparisons between egg volumes determined by the hematocrit and hemocytometer methods showed that the former method gave, on the average, results about 8 per cent higher than the latter This agrees with the recently reported results of Shapiro (21) Since the magnitude of this difference is known and reproducible, and since a considerable saving of time is effected the hematocrit method was employed through the seasons of 1934 and 1935 The values for oxygen consumption in both fertilized and unfertilized eggs are based on original unfertilized egg volumes in order to eliminate the fluctuations in volume which follow fertilization

In considering the suitability of these methods of handling and measurement, several facts may be emphasized (1) The rate of oxygen consumption in the control samples of eggs both fertilized and unfertilized, did not significantly increase or decrease during the usual 2 or 3 hour period of measurement This indicates that gaseous equilibrium was maintained and that no partial cytolysis of the eggs occurred (2) The rates of unit oxygen consumption obtained in different experiments did not deviate greatly from their average value even though they were obtained at different times during the seasons of 1934 and 1935 (3) Control samples of unfertilized eggs returned to sea water at the end of the period of measurement and then fertilized showed a high percentage of fertilization and subsequent development (4) Control samples of fertilized eggs, gave, during the course of respiratory measurement, 90 to 100 per cent regular cleavage On

return to sea water at the end of an experiment, almost all of the fertilized and dividing eggs in each control sample developed to normal swimming larvae

The data on division were obtained and expressed essentially by the method described by Smith and Clowes (22) According to this scheme, the unit is divisions per egg An egg which has divided to two cells has performed one division, an egg divided to four cells, two divisions, an egg divided to eight cells, three divisions, an egg divided to sixteen cells or more, four divisions Since, for eggs in optimum condition, about 65 minutes elapsed between fertilization and first cleavage, and about 30 minutes elapsed between successive subsequent cleavages, control eggs will have a division rate of about three at the end of a 2 hour experiment, and the maximum division rate of four, as defined above, at the end of a 3 hour experiment at 20°C After September 1st the division rates observed were somewhat smaller than these even when the temperature was maintained at 20°C

Each chemical was dissolved in fresh sea water just before use and the solution adjusted, if necessary, to the desired pH In all cases excepting those specifically mentioned the solutions were brought to pH 8 2, that of normal sea water In dissolving the phenols, it was found to be advantageous to add a few tenths of a cubic centimeter of normal sodium hydroxide directly to the weighed sample, to dilute with sea water almost to the required volume, and then to adjust the acidity Usually the solutions were added from the side arms of the manometer vessels to give the final concentrations shown in the experimental data Where the solubility of the chemical was too limited for the use of this method the solutions were added directly to the concentrated egg suspension before transfer to the Warburg vessels, using the necessary precautions to insure the experiments being comparable with those made at greater dilutions

The authors are indebted to Professor L F Fieser for generous supplies of 2,4-dinitro-α-naphthol and tetramethyl-p-phenylene diamine All other chemicals were prepared in the Lilly Research Laboratories or obtained in the purest available marketed form and suitably purified

Explanation of Figures

In Figs 1 and 2 the unit of oxygen consumption is cubic millimeters of O_2 per 10 c mm of eggs The unit of cell division is divisions per egg as defined in the previous section When the values for hourly oxygen consumption and the values for cell division are plotted against the logarithm of the concentration it is impossible to make the control points fall on the experimental curve Accordingly, these control values are represented by horizontal lines and labelled respectively, control O_2 and control division

For simplicity, and to bring out the relative effectiveness of various treatments, the data, in each figure subsequent to the first two, are presented on a relative basis The values of the two ratios,

$$\frac{O_2 \text{ consumed in treated eggs}}{O_2 \text{ consumed in control eggs}} \text{ and } \frac{\text{Cell division in treated eggs}}{\text{Cell division in control eggs}}$$

are expressed on the ordinate and the logarithm (base 10) of the molar concentration $\times 10^6$ on the abscissa   The oxygen consumption data in all figures subsequent to the first two may be converted to an absolute basis by noting that when the ratio is one, the oxygen consumption is equal to the control value   For all experiments given here, the control O value for fertilized *Arbacia* eggs at 20°C, and pH 8 2 may be taken as 3 1 ±0 3 c mm per hour per 10 c mm of eggs, this being the average of many experiments during the seasons of 1934 and 1935   At pH 6 5 and pH 7 (20°C) the control O_2 values are in the range 2 8 ±0 3 c mm per hour per 10 c mm of eggs   At other temperatures than 20°C, the control O values for fertilized eggs are At 12°C, 1 7 ±0 2, at 27°C, 5 5 ±0 5 c mm per hour per 10 c mm of eggs   The values at 12° and 27° are taken from duplicate determinations in a single experiment and are therefore subject to a greater possible variation than the control values at 20°C, which have been repeatedly determined

To compare the absolute and relative methods of presentation, it should be noted that the same set of data is plotted on an absolute basis in Fig 2, Part II, and on a relative basis in the 20°C curve of Fig 3

In all figures except Part I of Figs 1 and 2 circles are used for oxygen consumption data and crosses for cell division data   When the reagent has little or no effect on oxygen consumption or cell division (o nitrophenol, for example) the values for the two ratios given tend to fall at the unit level, when both crosses and circles are coincident, only one line is used to connect the points   In the cell division curves a distinction is made between reversible and irreversible division block, a solid line being used to denote the first and a dotted line being used to denote the second type of action

<div align="center">EXPERIMENTAL RESULTS</div>

Action of Dinitrocresol on Unfertilized Eggs —4,6-dinitro o cresol produces a rise in the rate of oxygen consumption by unfertilized *Arbacia* eggs (Fig 1)   The magnitude of this rise is dependent on the concentration of reagent, the optimum stimulation to some 600

per cent of the normal is obtained at a concentration of about 8×10^{-6} molar   There is a slight time lag before the optimum rate is established, but once reached, it is maintained for several hours without appreciable decrease

Although the cells are using their reserve material at a rate far in excess of the normal, these stimulated unfertilized eggs are not seriously injured by the reagent, and remain fertilizable for more than 24 hours (Table I), in fact, eggs treated with 10^{-4} molar 4,6-dinitro-*o*-cresol remain fertilizable for a longer period than the controls   When treated and untreated unfertilized eggs, which have been slowly shaken for many hours are transferred to sea water and fresh sperm added,

TABLE I

The Percentage Cleavage after Fertilization with Normal Sperm in Normal Sea Water of Eggs of Arbacia punctulata Which Have Previously Been Exposed to 4,6-Dinitro-o-Cresol at 20°C in the Following Molar Concentrations

Exposure	None	10^{-6}	5×10^{-6}	10^{-5}	10^{-4}
hrs					
4	95	95	95	95	95
8	95	95	95	95	95
12	85	90	95	95	95
15	68	70	90	90	94
18	23	45	45	60	90
21	20	20	20	40	60
24	5	5	5	20	20
28	0	0	0	0	10
32	0	0	0	0	0

the time elapsing between fertilization and first cleavage is nearly the same for the control and for the 4,6-dinitro-*o*-cresol treated eggs

It is known that many substances which induce artificial parthenogenesis can also stimulate the oxidative rate of unfertilized *Arbacia* eggs (see reference 24 for review)   Attempts to use the dinitro compounds as agents for inducing artificial parthenogenesis were unsuccessful

In view of the marked block to cell division described below for fertilized eggs, it should be recorded that the fertilization by sperm, as evidenced by successful membrane formation, can apparently take place in a concentration of $2 5 \times 10^{-3}$ molar 4,6-dinitro-*o*-cresol

Action of Nitrophenols and Related Compounds on Fertilized Eggs —
The nature of the respiratory and division effects produced by nitro
phenols and the environmental factors which influence the magnitude
of these effects may be illustrated by the typical results obtained with
4,6 dinitro *o* cresol   The first group of experiments is concerned with
the effect of various concentrations of reagent when added at a suitable
fixed time after fertilization   The oxygen consumption in any unit
of time is progressively increased up to concentrations of 5×10^{-6}
to 10^{-5} molar (Fig 2)   In the presence of concentrations greater
than these oxygen is taken up at a rate which is below the optimum,
and, at sufficiently high concentrations, below the normal   When the
data of Fig 1 for unfertilized eggs are compared with those of Fig 2
for fertilized eggs, the following facts are noted (1) The optimum
concentration of dinitrocresol is nearly the same in both cases   (2) At
this optimum the relative respiratory stimulation is greater for the
unfertilized than for fertilized eggs, the respective values being ap
proximately 600 and 300 per cent   Since the oxygen consumption of
normal fertilized *Arbacia* eggs is four times that of normal unfertilized
eggs, the optimum absolute rate in 4,6-dinitro *o* cresol treated fer
tilized eggs is approximately two and one half that of similarly treated
unfertilized eggs   (3) The oxygen consumption of fertilized eggs is
depressed below the normal at concentrations of dinitrocresol above
2.5×10^{-4} molar   That of unfertilized eggs is almost twice the normal
at 5×10^{-4} molar, and, at considerably higher concentrations, does
not fall below the normal until the eggs are destroyed   The signifi
cance of the falling oxygen consumption with increasing concentra
tion of reagent will be considered at a later point in this paper

In eggs treated, at 20°C , with 4,6 dinitro *o* cresol within 25 minutes
after fertilization the first and subsequent cell divisions are unimpaired
until that concentration of reagent which is optimum for respiration
is attained   At this concentration of dinitrocresol, the number of
divisions performed by the eggs is less than the number of divisions
in the control eggs   In a slightly higher concentration of dinitrocresol,
the eggs do not divide at all   As shown by the continuous line of the
division curve this block to division is reversible after many hours
exposure to concentrations almost 500 times those which first produced
the division block   On return to sea water, the eggs resume division

at the normal rate and develop, after the usual interval, to swimming larvae   In this, and all subsequent experiments with stimulating nitrophenols, the block to division invariably commences at or near
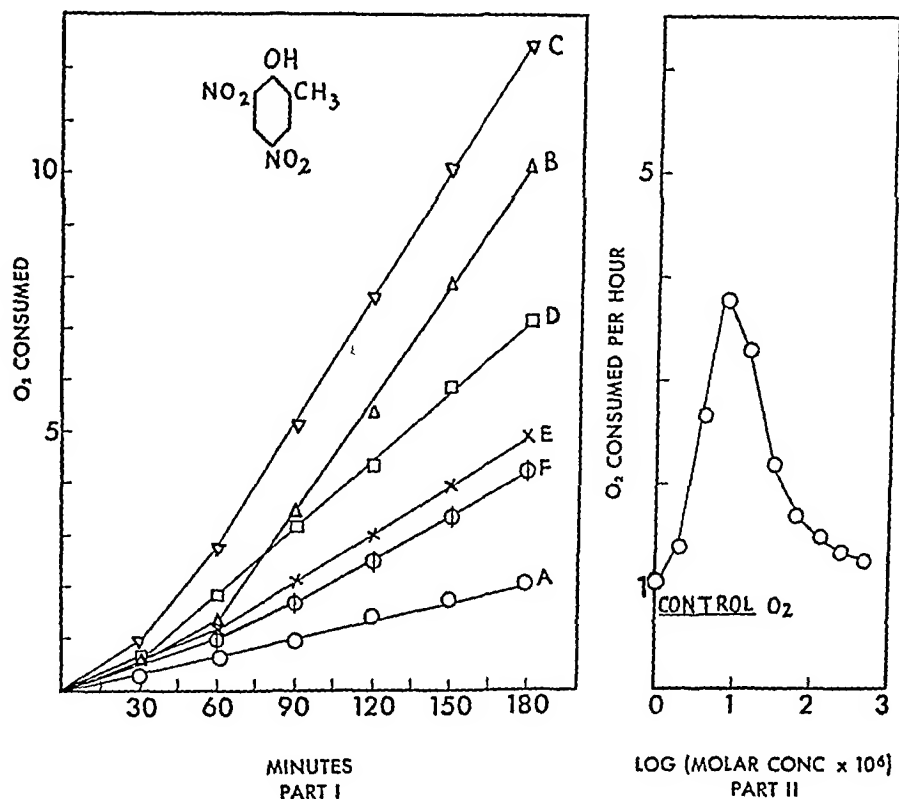
FIG 1   Part I   Oxygen consumption, at 20°C , of unfertilized *Arbacia* eggs for various periods of time in the following molar concentrations of 4,6-dinitro-*o*-cresol   *A*, none-control, *B*, 4×10^{-6}, *C*, 8×10^{-6}, *D*, $3\,2 \times 10^{-5}$, *E*, $1\,28 \times 10^{-4}$, *F*, $5\,12 \times 10^{-4}$

Part II   Hourly oxygen consumption, at 20°C , of unfertilized *Arbacia* eggs in various concentrations of 4,6-dinitro-*o*-cresol

the peak and continues throughout the down curve of oxygen consumption

Shortly after these experiments were first reported (6), Martin and Field (31) described experiments showing that, in concentrations greater than the respiratory optimum, 2,4-dinitrophenol had an adverse effect on the multiplication of yeast (see also references 9, 19)

A second group of experiments is concerned with the effect of a fixed concentration of reagent added at various times after fertilization The effect on oxygen consumption is nearly independent of the time
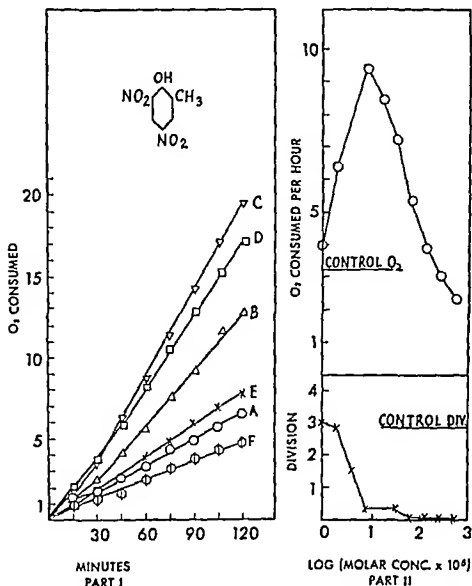
FIG 2 Part I   Oxygen consumption at 20 C , of fertilized *Arbacia* eggs for various periods of time in the following molar concentrations of 4 6-dinitro-*o*-cresol *A* none-control *B* 2 × 10⁻⁵ *C*, 4 × 10⁻⁵ *D* 1 6 × 10⁻⁵ *E*, 1 28 × 10⁻⁴ *F*, 5 12 × 10⁻⁴

Part II   Hourly oxygen consumption and cell division, at 20°C , of fertilized *Arbacia* eggs in various concentrations of 4 6-dinitro-*o*-cresol

elapsing between fertilization and addition of reagent, while the block to division is sharply dependent on this factor (Table II)   At 20°C , there is a critical point 20 to 25 minutes before first cleavage is

scheduled to occur   If dinitrocresol in appropriate concentration is added prior to this time relatively few eggs divide, if the reagent is added after this time, a normal proportion of the eggs proceeds to the two cell stage, where most of the eggs are arrested in development   A similar change in sensitivity is observed shortly before the second and third cleavages

From data concerning the effect of various concentrations of dinitro-cresol at temperatures of 12°C , 20°C , and 27°C (Fig 3), it may be seen that   (1) The concentration which is optimum for oxidative stimulation and critical for initial division block does not vary greatly as the temperature is changed, (2) at the optimum concentration the

TABLE II

The Effect of 8 × 10⁻⁶ Molar 4,6-Dinitro-o-Cresol on Oxygen Consumption and Division in Fertilized Eggs of Arbacia punctulata at Various Times after Fertilization   Temperature 21°C   pH 8 2

Time of addition after fertilization	O₂ consumed c mm per hr per 10 c mm eggs	Divisions per egg 140 min after fertilization
min		
20	10 7	0 10
30	10 8	0 20
40	10 7	0 95
50	10 8	0 95
55	10 7	0 95
No addition	2 9	2 95

Control 50 per cent divided to 2 cell at 62 minutes   Eggs 98 per cent fertilized

oxygen consumption of the treated eggs, as compared with that of the untreated control eggs, decreases as the temperature is raised, while the absolute number of units of excess oxygen consumed is least at 12°C , rises somewhat between 12°C and 20°C , and remains prac-tically constant between 20°C and 27°C , (3) the curves relating the oxygen consumption and the logarithm of concentration are the same in general form and differ only slightly as to the slope of their ascending and descending portions

The 4,6-dinitro-*o*-cresol effect on respiration and cell division in *Arbacia* eggs is not particularly sensitive to changes in hydrogen ion concentration (Fig 4)   At 20°C , the optimum concentration of

dinitrocresol is slightly greater at pH 8 2 and pH 7 than at pH 6 5
The blocking effect of any given concentration on cell division is
slightly greater at pH 6 5 than at lower acidity

Accompanying the increase in oxygen consumption, there is a simul
taneous and nearly equivalent increase in CO_2 production   The ratio

LOG (MOLAR CONCENTRATION x 10⁶)

Fig 3 Stimulation of oxygen consumption and block to cell division in fer
tilized *Arbacia* eggs produced by various concentrations of 4 6-dinitro-*o*-cresol at
12 C 20 C , and 27°C   Reagent added 15 minutes after fertilization
  In Figs 3–10

$$O - O = \frac{O_2 \text{ consumed in treated eggs}}{O \text{ consumed in control eggs}}$$

$$\times - \times = \frac{\text{Cell division in treated eggs}}{\text{Cell division in control eggs}}$$

CO_2 produced/O_2 consumed is not significantly influenced by dinitro-cresol, even when the oxygen consumption is raised to three or more times the normal level   The values of the respiratory quotient for dinitrocresol treated eggs (Table III) are slightly higher than the
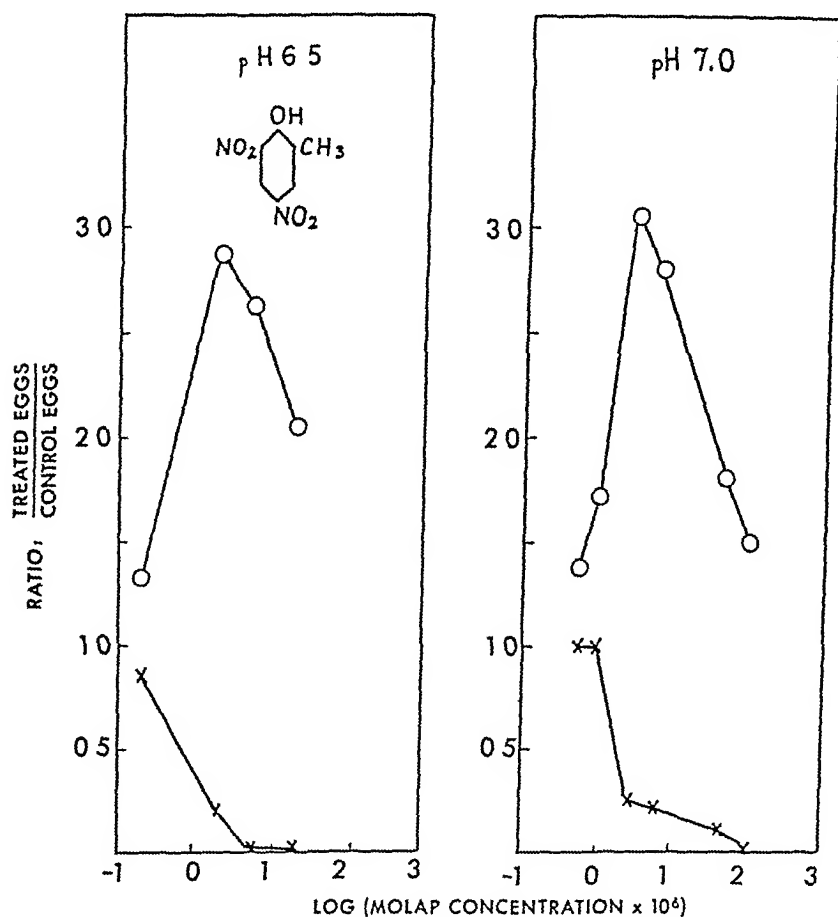
FIG 4 Stimulation of oxygen consumption and block to cell division in fertilized *Arbacia* eggs produced by various concentrations of 4,6-dinitro-*o*-cresol at pH 6 5 and pH 7   Temperature, 20°C   Reagent added 25 minutes after fertilization

normal values obtained in the present work and the normal values for eggs of sea urchins of other species (20, 23)

If, with a constant concentration and volume of 4,6-dinitro-*o*-cresol solution, varying numbers of eggs are employed, the unit oxygen con-

TABLE III

Respiratory Quotient of Fertilized Eggs of Arbacia punctulata When Stimulated by 8×10^{-8} Molar, 4 6 Dinitro-o Cresol   Temperature 21 C

Flask No.	CO₂ (initial)	CO₂ (total)	CO₂ (produced)	O₂ (consumed)	R Q
	c mm	*c mm*	*c mm*	*c mm*	
1	297				
2		499		218	
3	290				
4		508		221	
5	294				
6		497		217	
Average	294	501	207	219	0 94

TABLE IV

Respiratory Stimulation in Fertilized Eggs of Arbacia punctulata with Fixed Concentration and Volume of 4,6 Dinitro o Cresol Solution and Varying Concentrations of Eggs   Temperature 20°C

No of eggs	O₂ Consumed (c.mm per hr per 10 c.mm eggs)	
	Control	8×10^{-8} m lar 4 6-dinitro-o cresol
per cc		
58 000	3 14	9 04
116 000	2 94	9 03
232 000	2 83	9 09

TABLE V

Block to cell division in fertilized eggs of *Arbacia punctulata* with a fixed con centration and volume of 4 6-dinitro o cresol solution and varying concentrations of eggs   The reagent was added at 25 minutes after fertilization and the eggs were not shaken   Temperature 20 C

No f eggs	Divisions per egg after 3 hrs exposure	
	Control	$1 6 \times 10^{-8}$ molar 4 6-dinitro-o cresol
per c		
300	> 4	3 38
600	> 4	3 41
1200	> 4	2 88
2400	> 4	1 83

sumption in the normal and in the treated eggs does not vary significantly with the egg concentrations (Table IV)   Furthermore, under these conditions, a partial division block is not made more complete
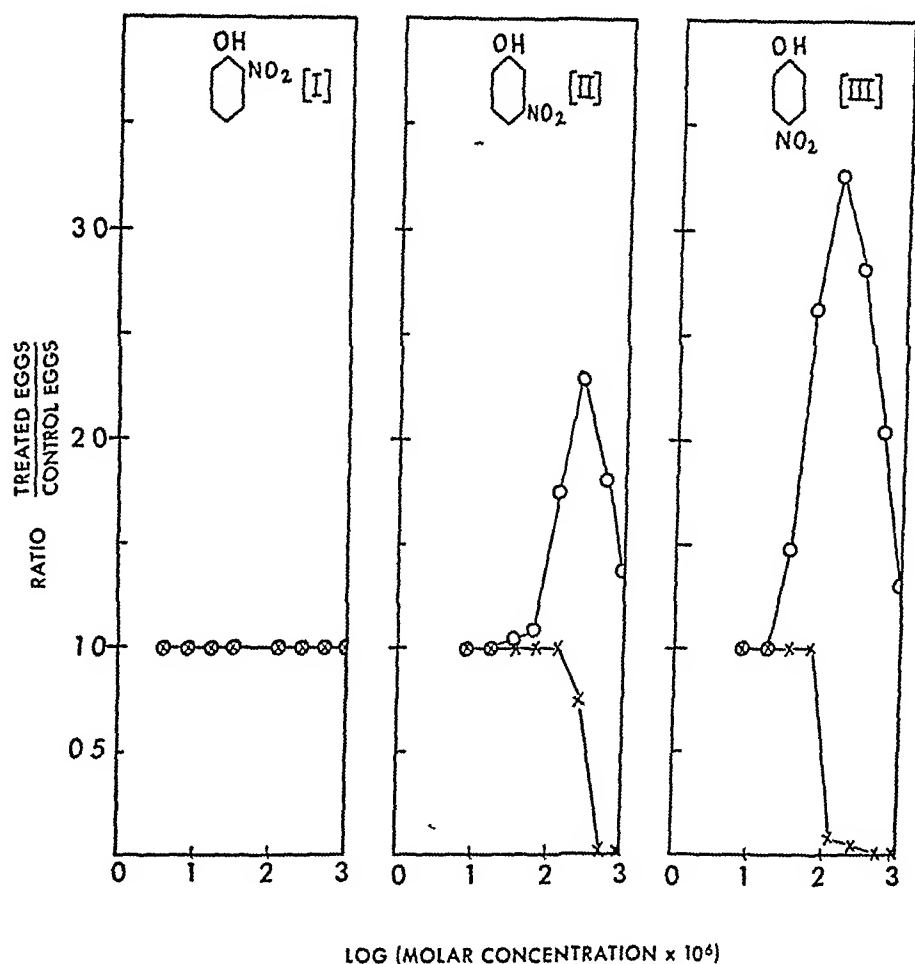
LOG (MOLAR CONCENTRATION x 10⁶)

FIG 5 Stimulation of oxygen consumption and block to cell division of fertilized *Arbacia* eggs produced by various concentrations of *o*-nitrophenol (I), *m*-nitrophenol (II), and *p*-nitrophenol (III) at 20°C   Reagents added 15 minutes after fertilization

as the number of eggs is decreased (Table V)   In fact, the division block is somewhat less when smaller numbers of eggs are used, possibly due in part to the less rapid accumulation of metabolic products in the more dilute suspensions   In this fact there is some support for

the hypothesis that the division block by 4,6-dinitro-o cresol may be due, in some degree, to the ability of this reagent to stimulate anaerobic production of metabolic intermediates unfavorable for divi-
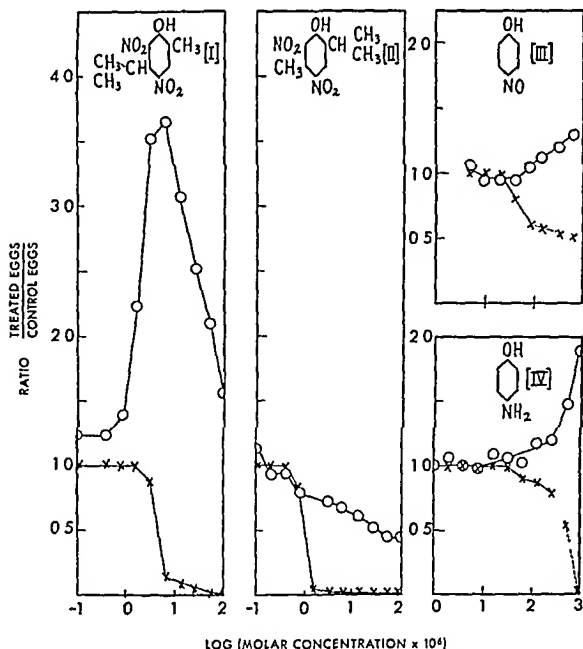
FIG 6 Stimulation of oxygen consumption and block to cell division of fertilized *Arbacia* eggs produced by various concentrations of 4 6-dinitrocarvacrol (I), 2 4-dinitrothymol (II) *p* nitrosophenol (III) and *p* aminophenol (IV) at 20°C Reagents added 15 minutes after fertilization

sion (15) The rate of intracellular accumulation of such intermediates, and the division block resulting therefrom, may decrease as the egg concentration is made smaller

The simplest compounds containing the phenol nucleus and a nitro group are the three isomeric mononitrophenols   The o-nitrophenol does not stimulate oxygen consumption or block division in the eggs until very high and permanently injurious concentrations are reached

LOG (MOLAR CONCENTRATION x 10^6)
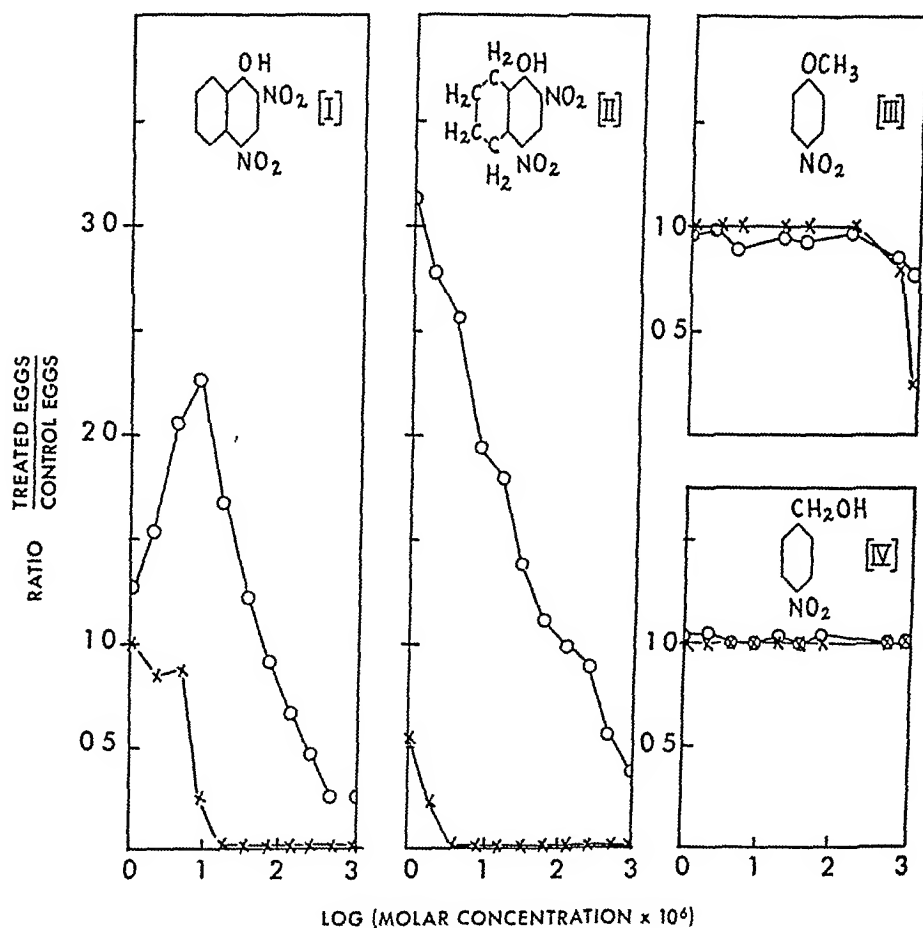
Fig 7 Stimulation of oxygen consumption and block to cell division of fertilized *Arbacia* eggs produced by various concentrations of 2,4-dinitro-α-naphthol (I), 2,4-dinitro-*ar*-tetrahydro-α-naphthol (II), p-nitroanisole (III), and p-nitrobenzyl alcohol (IV) at 20°C   Reagents added 15 minutes after fertilization

The m- and p-nitrophenols stimulate oxygen consumption and block division in the same general way, but to a lesser degree than 4,6-dinitro-o-cresol   The p-nitrophenol is considerably more active than the m-nitrophenol in raising respiration, and in suppressing division

(Fig 5) The *p* nitrophenol is more active than any compound in which the nitro group in the para position has been replaced by another atom or radical For example, *p* nitrosophenol and *p* aminophenol, derived from the *p* nitrophenol by reduction, have no marked ability
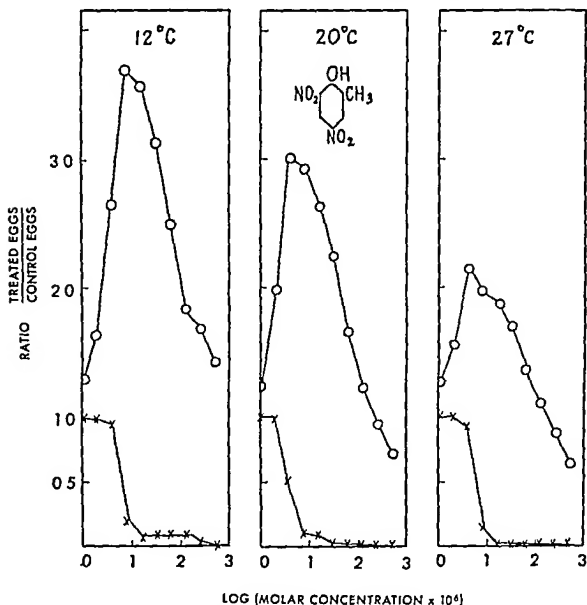

FIG 8 Stimulation of oxygen consumption and block to cell division of fertilized *Arbacia* eggs produced by various concentrations of 2,4-dinitrophenol (I) 2 6-dinitrophenol (II), 2 4-dinitroaniline (III), and 2 4 6-trinitrophenol (IV) at 20°C Reagents added 15 minutes after fertilization

to stimulate oxygen consumption or reversibly block division in the range of concentrations effective for nitro compounds (Fig 6) When other groups are substituted for hydroxyl, even in the favorable para position to the nitro group, the ability to stimulate respiration and

block division is lost   The substitution of a methyl group for the phenolic hydrogen, as in p-nitroanisole (Fig 7), or the separating of the hydroxyl from the ring by a $-CH_2$ group, as in p-nitrobenzyl alcohol, destroys the activity (Fig 7)

Proceeding from the mononitrophenols and their derivatives to the dinitro compounds, it is found that 2,4-dinitrophenol is more active than 2,6-dinitrophenol in stimulating oxygen consumption and blocking division (Fig 8)

Field, Field, and Martin (27) have found that the relative effectiveness of these compounds in producing stimulation of oxygen consumption in yeast is as follows p-nitrophenol > m-nitrophenol > o-nitrophenol, 2,4-dinitrophenol > 2,6-dinitrophenol

Introduction of the methyl group into the nitrophenol nucleus in the ortho position to the OH results in greater effectiveness at lower concentrations, for example, the optimum for 4,6-dinitro-o-cresol is 8×10^{-6} molar as compared with the optimum of $3\,2 \times 10^{-5}$ molar for 2,4-dinitrophenol

If two aliphatic side chains are introduced into the 2,4-dinitrophenol molecule the activity of the resulting compound depends on the relation of the alkyl groups to the hydroxyl and to the nitro groups   For instance there is a remarkable difference between 4,6-dinitrocarvacrol (1-methyl, 2-hydroxy, 4-isopropyl, 3,5-dinitrobenzene), which stimulates oxygen consumption and blocks division in the same way as 2,4-dinitrophenol, and 2,4-dinitrothymol (1-methyl, 3-hydroxy, 4-isopropyl, 2,6-dinitrobenzene) which blocks division in extremely low concentrations with only a slight stimulation of oxidation (Fig 6) A full discussion of the significance of this difference must be reserved for a later paper   The fact that 2,4-dinitrothymol fails to stimulate respiration and yet blocks division in a concentration of 10^{-6} molar, added to the fact that 4,6-dinitrocarvacrol and other nitrophenols blocking division at a concentration of 10^{-5} molar or more exhibit respiratory stimulus with a down curve of oxygen consumption coinciding with the block to division, suggests that the biological effects of these agents may be the result of two factors   One factor leads to stimulation of oxygen consumption and does not affect cell division The other factor leads to a limitation of the rate of oxygen consumption and suppression of cell division

Enlarging the carbocyclic grouping to which the hydroxyl and nitro groups are attached tends, in some cases, to increase the activity at low concentrations   As examples, 2,4 dinitro α naphthol and 2,4-dinitro ar tetrahydro α naphthol may be compared with 2,4-dinitro phenol (Fig 7)

TABLE VI

Nitro and related compounds which have little or no effect on the cell division of fertilized eggs of *Arbacia punctulata* when the eggs are first exposed at either 15 or 40 minutes after fertilization at 20°C

Compound	Concentration giving the following results after a 3 hrs exposure			
	Cytolysis	Irreversible block	Reversible block	No effect
	moles per l	*moles per l*	*moles per l*	*moles per l*
o-Aminophenol	10^{-2}	10^{-4}		10^{-5}
o Nitroanisole				10^{-2}
p-Nitrophenetole			10^{-2}	10^{-4}
p-Nitrobenzonitrile				10^{-2}
p Nitroacetonitrile				10^{-2}
p-Nitrophenylacetic acid				10^{-2}
p Nitrobenzamide				10^{-2}
p Nitrophenylisocyanate				10^{-2}
o Nitroaniline				10^{-2}
p-Nitroaniline				10^{-2}
p-Nitrodimethylaniline				10^{-2}
p-Nitrodiethylaniline		10^{-2}		10^{-4}
p-Nitrosodimethylaniline	10^{-2}			10^{-4}
p-Nitrosodiethylaniline	10^{-2}	10^{-4}		10^{-6}
o-Dinitrobenzene	Insoluble			
p-Dinitrobenzene	10^{-2}			10^{-4}
o-Nitrochlorobenzene			10^{-2}	10^{-4}
m Nitrochlorobenzene				10^{-2}
p-Nitrochlorobenzene			10^{-2}	10^{-4}

As in the mononitro derivatives, replacement of the hydroxyl in 2,4-dinitrophenol by an amino group to give 2,4 dinitroaniline results in almost complete loss of activity (Fig 8)

It is particularly interesting to note that when a third nitro group is introduced into the highly active 2,4-dinitrophenol or 2,6 dinitro phenol to form 2,4,6 trinitrophenol (picric acid) there is a total loss of activity (Fig 8)

Other nitro and related compounds found to be ineffective in producing a marked respiratory stimulation or a division block are listed in Table VI It should be particularly noted that dinitrobenzene and other nitro compounds which do not contain the phenolic hydroxyl group exert no effect on cell division until very high and usually lethal concentrations are reached

Action of Reversible Dyes on Fertilized Eggs —Certain substances capable of being reduced in the cell and reversibly reoxidized by oxidiz-
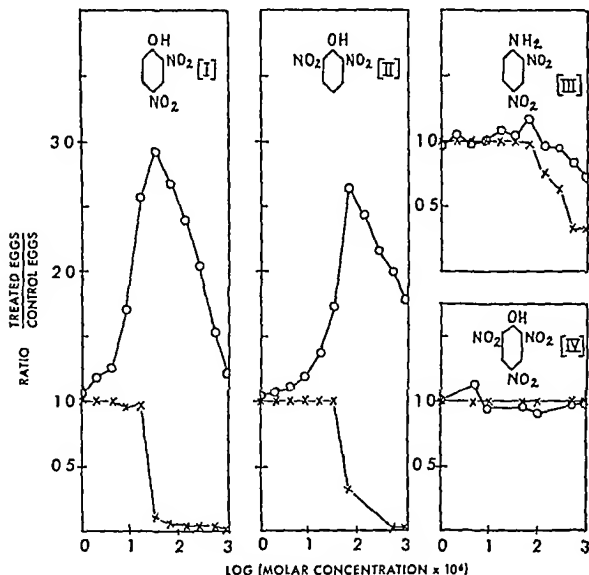

LOG (MOLAR CONCENTRATION x 10⁶)
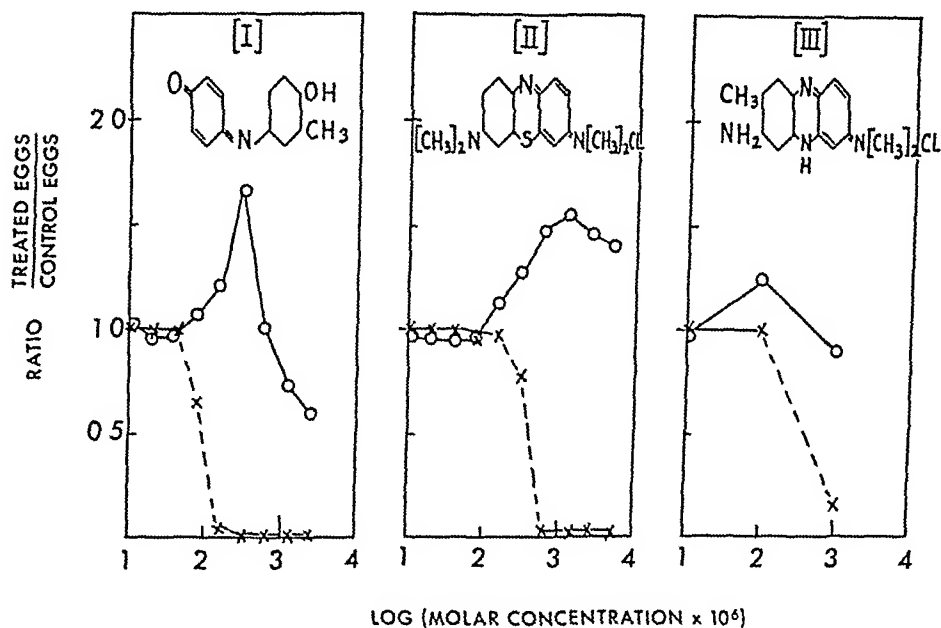
Fig 9 Stimulation of oxygen consumption and block to cell division of fertilized *Arbacia* eggs produced by various concentrations of *o*-cresol indophenol (I), methylene blue (II), and neutral red (III) at 20°C The dotted division lines denote irreversible injury Reagents added 15 minutes after fertilization

ing agents, including oxygen, produce, when present in relatively low concentrations, a rise in rate of oxygen consumption of marine eggs

Methylene blue, *o*-cresol-indophenol, and neutral red, three oxidation-reduction indicators of potentials ranging from $E_0' = +200$ mv to -300 mv at pH 7 (the intracellular pH) and 30°C (4, 16), raise oxygen consumption to varying degrees With none of these reagents is the stimulation of oxygen consumption as great as with the nitro and dinitrophenols (Fig 9) In relatively high concentration the

reversible dyes block division   This division block is, to a large extent, irreversible, since the eggs do not recover on return to sea water   It

LOG (MOLAR CONCENTRATION x 10⁶)
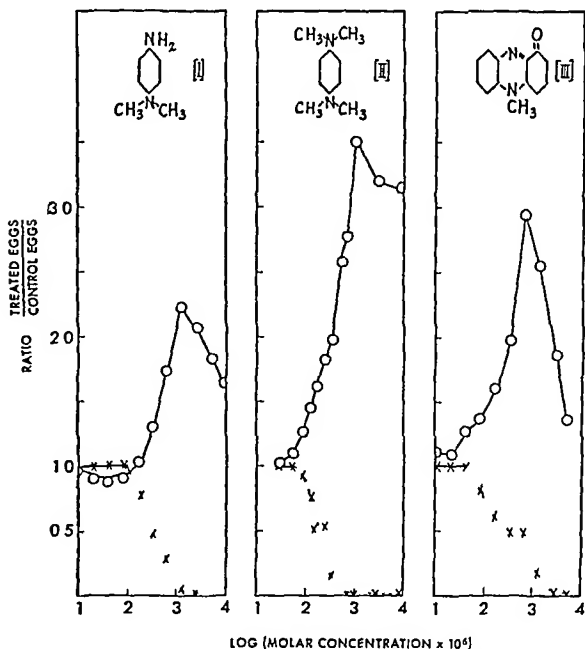
FIG 10 Stimulation of oxygen consumption and block to cell division of fertilized *Arbacia* eggs produced by various concentrations of dimethyl *p* phenylene diamine (I), tetramethyl *p* phenylene diamine (II) and pyocyanine (III) at 20 C The dotted division lines denote irreversible injury   Reagents added 15 minutes after fertilization

is desired to emphasize the fact that fertilized eggs of *Arbacia* subjected to optimum respiratory stimulating concentrations of these dyes are, so far as subsequent division is concerned, dead cells

When a fixed concentration of each of the three above reagents is added separately to samples of the fertilized eggs at varying times during the first and later mitotic cycles no particular variation in intensity of division block is observed   If the concentration is large enough to produce a total block to division the eggs do not perform further divisions regardless of the time in the division cycle at which the reagent is added   This is in marked contrast to the action of 4,6-dinitro-o-cresol and certain other respiratory stimulating nitrophenols, to which *Arbacia* eggs are particularly sensitive prior to one certain point in each mitotic cycle

Three two step reversible oxidation-reduction indicators, dimethyl-*p*-phenylene diamine, tetramethyl-*p*-phenylene diamine, and pyocyanine also increase respiration and irreversibly block division in higher concentration (Fig 10)   The first two have values for E_0' at pH 7 and 30°C , in the range $+250$ mv to $+350$ mv   The latter has an E_0' at pH 7 and 30°C , of the order of -30 mv (17)

After immersion of the eggs in dimethyl-*p*-phenylene diamine there is a marked decrease in the intensity of color of the dye solution Since the eggs produce a similar decrease in the color of *o*-cresol-indophenol it may safely be assumed that, under the present experimental conditions, the eggs can reduce these two dyes of high potential (see Chambers, Pollack, and Cohen (2))   This reduction, when the dye concentration is 10^{-4} molar or less, results in no interference with the division rate of the eggs

DISCUSSION

The conclusion has been reached that the mode of action of nitrophenols and related substances differs essentially from that of methylene blue, pyocyanine, or other oxidation-reduction indicators   The most significant facts bearing on this point may be summarized as follows

1   4,6-dinitro-o-cresol and other active nitro compounds give optimum stimulation of respiration and initial reversible block to division at concentrations as low as 10^{-6} molar, while the oxidation-reduction dyes exert their maximum effect on oxygen consumption in the range 5×10^{-4} to 5×10^{-3} molar, a concentration one hundred to one thousand times as great as that at which most of the active nitro compounds are effective

2 The block to division exerted by nitrophenol derivatives close to the peak of respiratory stimulus is fully reversible, the eggs developing in a normal manner when returned to sea water This block to division remains fully reversible until concentrations one hundred to five hundred times those required for initial division block are reached The oxidation reduction dyes, when present in sufficient concentration to produce significant rises in oxygen consumption, exert a destructive effect since the eggs so treated do not divide when returned to sea water after a 3 hour exposure

3 The partial reduction products of nitro compounds such as *p* nitrosophenol and *p* aminophenol, are in no way comparable to the nitro compounds in their ability to stimulate respiration or reversibly block cell division

4 No effect on oxygen consumption or cell division results from the intracellular reduction of an amount of reversible dye more than ten times the equivalent of the amount of nitro compound present in a respiratory stimulating and division blocking solution of the latter

5 The optimum concentration of 4,6-dinitro *o* cresol and the absolute amount of excess oxygen consumed at this optimum concentration change only slightly with temperature

6 The *Arbacia* eggs show a cyclic sensitivity to reversible division block by certain nitro compounds, the division block by dyes, which in any case is irreversible, appears to be equally intense at all points in the mitotic cycle

7 4,6-dinitro *o* cresol raises oxygen consumption in both fertilized and unfertilized eggs of *Arbacia punctulata* Under optimum stimulation the absolute level in fertilized eggs is two and one half to three times that in unfertilized eggs This result contrasts with that obtained by Runnstrom (20) with dimethyl *p* phenylene diamine, one of the two step oxidation reduction indicators This latter substance raises the rate of oxygen consumption to approximately the same level in both fertilized and unfertilized *Arbacia* eggs

In forming a working hypothesis to replace or supplement the one based on oxidation reduction, there are several suggestive facts to consider First, the division blocking effect of 4,6-dinitro *o* cresol is rapidly and completely reversible Second, little or no destructive action results from long exposure of the unfertilized eggs to the

reagent   Third, there is no evidence of a direct relationship between
egg numbers and the respiratory stimulating and division blocking
effects produced by a given amount of a given concentration of reagent
The reversible combination of the 4,6-dinitro-*o*-cresol with some cel-
lular constituent, or the adsorption of the reagent on and displacement
of substrate from one or more enzymatic surfaces might well be asso-
ciated with such a dependence on the concentration and independence
of the total amount of reagent employed   A fourth point is that the
decrease in 4,6-dinitro-*o*-cresol stimulation of respiration with rising
temperature is in harmony with a concept involving reversible com-
bination or adsorption

A fifth point in favor of accepting such a concept as a working hy-
pothesis is found in the fact that the nitrophenols are known to form
molecular addition compounds with nitrogen containing substances
similar to those which are known to play a rôle in cellular metabo-
lism (18)   In the case of the isomeric mononitrophenols, the tendency
toward the formation of such addition compounds parallels the ability
to stimulate respiration and block division, being greatest for *p*-nitro-
phenol and least for *o*-nitrophenol   In this connection, the possibility
of formation of nitrophenol-metal complexes may also be of impor-
tance   It has also been suggested that the dinitrophenols may com-
bine with a substance acting as a regulating factor for respiration (7)
or may bring new, previously unused, respiratory centers into play (1)

Handovsky and coworkers (28) have emphasized the reduction of
nitro compounds by cells and have also stressed the effects of these
compounds on the reducing activity of tissues and tissue extracts
Alwall (25) has suggested that the dinitrophenols act as reversible
oxidation-reduction systems which aid in the transport of hydrogen
to the cytochrome-oxidase system

If some reversible combination of the sort mentioned here does
occur, the experiments presented in this paper may perhaps be used
to indicate by analogy the type of attractive forces involved   (*a*)
Combination cannot be due to any unsaturated affinity associated
with the nitro groups, since picric acid is inactive in raising respiration
or blocking division at pH 8 2   (*b*) The acidity of the phenol group
is not an important factor, since *o*-nitrophenol has a pK of approxi-
mately 7 2, *p*-nitrophenol a pK of approximately 7 2, 2,4-dinitro-

phenol a pK of approximately 4 0 (3) The former substance is inactive up to very high concentrations and the latter two substances are very active in stimulating respiration and blocking division (c) The high dipole moment, and consequent additive properties of the entire molecule are not likely to be involved, since p nitroaniline and p nitro somethylamilne, which have no effect on respiration or division, have dipole moments equal to or greater than that of p nitrophenol (11) (d) Any compounds formed are not likely to be of the oxonium type, since the mixed ether, p nitroanisole, produces in low concentrations no rise in oxygen consumption or block to division

Since the phenol hydroxyl, from the experiments to date, appears to be indispensable, the free hydroxyl group is suggested as the principal center of combination

When the effects of a given compound on respiration are compared with the effect of the same compound on cell division, it is observed that the range of concentration in which the reversible division block is produced coincides with the range of concentration in which the rate of respiration is decreasing from the optimum value This relation ship holds for a single compound at different temperatures and for a wide variety of compounds at a single temperature Hence, the reversible division block seems to be associated with a limitation, disturbance, or block of an aerobic phase of metabolism rather than with an actual oxygen lack The behavior of the two isomers, 4,6 dinitrocarvacrol and 2,4-dinitrothymol supports this view The former gives a block to division and a respiratory stimulation to some 350 per cent of the normal at a concentration of about 10^{-5} molar, the latter gives a reversible block to division at an extraordinary dilution of about 10^{-6} molar with little or no previous or coincident rise or fall in respiratory rate

The coincidence of the down curve of respiration and the reversible block to division by nitro compounds in *Arbacia* eggs might well be the result of (1) Activation or inactivation by the nitro compound of one or more substances concerned with metabolism and cell division, or (2) accumulation, in high local concentration, of an intermediate metabolite unfavorable to division From the evidence available, the former of these two hypotheses appears the more probable

The authors desire to thank Miss A K Keltch for her valuable assistance during this investigation, particularly for procuring the data given in Table I

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STUDIES ON CELL METABOLISM AND CELL DIVISION

II STIMULATION OF CELLULAR OXIDATION AND REVERSIBLE INHIBITION OF CELL DIVISION BY DIHALO AND TRIHALOPHENOLS

BY M E KRAHL AND G H A CLOWES

(From the Lilly Research Laboratories, Marine Biological Laboratory, Woods Hole)

Two types of reagents have previously been known to produce large increases in the rate of oxygen consumption by living cells   These are, first, certain oxidation reduction indicators (2), and, secondly, certain nitro and dinitro derivatives of phenols (7)   (See also Krahl and Clowes (6) and Alwall (1) for numerous other references)   In this paper it will be shown that certain dihalo and trihalophenols produce, in fertilized sea urchin eggs (*Arbacia punctulata*), a rise in oxygen consumption and a reversible block to cell division comparable to those effected by nitro and dinitrophenols and differing from those obtainable with the oxidation reduction indicators of the type of methylene blue

On the basis of experimental results presented in a previous paper (4), it was tentatively concluded that the metabolism stimulating and reversible division blocking properties of the nitrophenols were not the consequence of reduction or reversible oxidation of the nitrophenol molecule   Since the dihalo and trihalophenols display the same oxidation promoting quality and nearly the same reversible division suppressing ability as the nitro and dinitrophenols, it is reasonable to believe that all these phenol derivatives have a common mode of action on the cell

In a dihalo or trihalophenol molecule there are no substituent groups capable of reduction, or reversible oxidation reduction, and the entire halogen substituted phenol molecule is oxidized only irreversibly and at very high positive potentials (5)   Hence the present experiments provide further confirmation of the hypothesis previously advanced

According to this view, the dinitrophenols and the dihalophenols derive their intense biological activity from the presence of the phenolic OH group, as modified by suitable substitution in the benzene ring
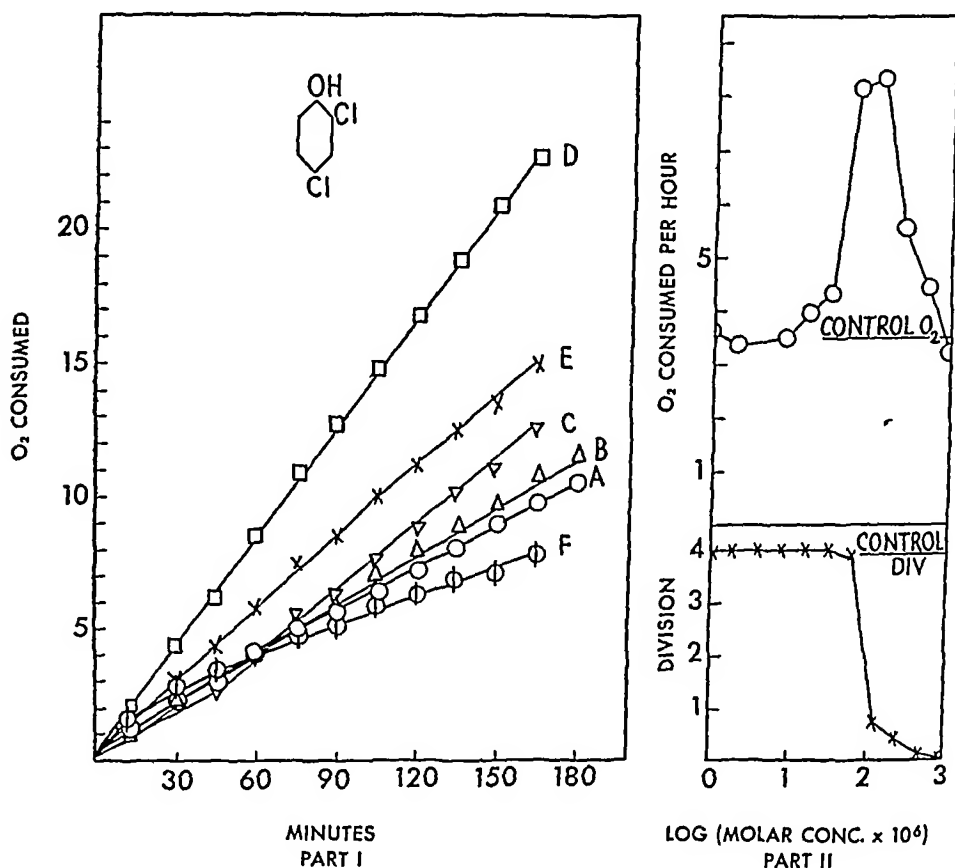
FIG 1   Part I   Oxygen consumption, at 20°C , of fertilized *Arbacia* eggs for various periods of time in the following molar concentrations of 2,4-dichlorophenol *A*, none-control, *B*, 1 6 × 10⁻⁵, *C*, 3 2 × 10⁻⁵, *D*, 1 28 × 10⁻⁴, *E*, 2 56 × 10⁻⁴, *F*, 1 024 × 10⁻³

Part II   The hourly oxygen consumption and the cell division, at 20°C , of fertilized *Arbacia* eggs in various concentrations of 2,4-dichlorophenol   Reagent added 25 minutes after fertilization

Although this concept can be made to explain all of the experimental facts available, and although it has led to the realization that the dihalophenols should and do act as cellular oxidative stimulants, further elaboration of the idea will be reserved until the theoretical

consequences of such a concept can be tested by further experi
ments

The present experiments were performed and the results expressed
by the use of the methods described in a previous paper (4) Ferti
lized eggs of the sea urchin (*Arbacia punctulata*) were employed

Fig 2 Stimulation of oxygen consumption and block to cell division of fer
tilized *Arbacia* eggs produced by various concentrations of 2 4-dichlorophenol
(I), 2 4-dibromophenol (II), and 2 6-dibromophenol (III) at 20 C Reagent
added 15 minutes after fertilization

In Figs 2–6

$$O — O = \frac{O_2 \text{ consumed in treated eggs}}{O \text{ consumed in control eggs}}$$

$$\times — \times = \frac{\text{Cell division in treated eggs}}{\text{Cell division in control eggs}}$$

EXPERIMENTAL RESULTS

To illustrate the behavior of the dihalo and trihalophenols, the
results with 2,4-dichlorophenol may be cited in detail When, at a

given time after fertilization, various concentrations of this reagent are added to samples of fertilized and developing eggs of *Arbacia*, the following results are obtained

Within a short time after the addition of a suitable single stimulating concentration of the reagent, the rate of oxygen consumption by the eggs is increased to more than twice the normal value   This increased rate is maintained for at least 3 hours (Fig 1)

The rate of oxygen consumption (which is here expressed as the hourly average of a 2 hour observation) depends on the concentration of reagent   As this concentration is increased from zero, the rate of oxygen consumption by the treated eggs increases until an optimum rate is attained   At still higher concentrations the rate of oxygen consumption is less than at this optimum

If a concentration of 2,4-dichlorophenol slightly greater than the optimum for respiration is added within 30 minutes after fertilization, at 20°C, the eggs do not divide while the untreated control eggs proceed to the 16 cell stage

90 to 100 per cent of all fertilized eggs which have been completely blocked in division by 2,4-dichlorophenol resume their mitotic activity immediately after return to sea water and develop to swimming larvae This holds true for exposures of 3 hours and concentrations up to 10^{-3} molar, the latter concentration being ten to twenty times the optimum for respiration   This reversibility of the division blocking effect in concentrations greater than the respiratory optimum deserves particular emphasis because it is displayed by the respiratory stimulating nitro compounds and is not shown by any of the respiratory stimulating oxidation-reduction dyes which have so far been investigated (4)

A second general question of interest, in view of the results previously obtained with nitro compounds (3, 4) is that of variation in intensity of block to cell division obtainable by varying the time after fertilization at which a suitable fixed concentration of reagent is added to a sample of eggs   As in the case of the nitro compounds, it is found that the division block is greatest when the reagent is added within thirty minutes after fertilization   If the reagent is added at subsequent times during the first mitotic cycle, the eggs can complete the first division but do not perform the second division (Table I)   This

cyclic variation in sensitivity to 2,4-dichlorophenol is repeated in the second mitotic cycle of fertilized *Arbacia* eggs

An experimental survey of a number of other dihalophenols and their derivatives brings out the following data concerning the dependence of biological activity on the type and arrangement of the substituent groups in the phenol molecule

First, the introduction of a methyl group into dichlorophenol does not markedly affect the activity, since 4,6 dichloro *o* cresol is approximately equal to 2,4-dichlorophenol in regard to its effective concen

TABLE I

The Effect of 10^{-4} Molar 2 4-Dichlorophenol on Division in Fertili ed Eggs of Arbacia punctulata at Various Times after Fertili ation   Temperature $19°C$, and pH 8 2

Time of addition after fertilization	Divisions per egg at additi n of reagent	Divisions per egg 180 min. after fertilization
m n		
10	0	0 02
20	0	0 12
30	0	0 14
40	0	0 43
50	0	0 83
60	0 05	0 90
65	0 30	0 94
70	0 90	0 95
75	0 97	1 00
No addition	—	4 00

tration and in regard to the percentage effect produced (Fig 3)   The presence of aliphatic side chains in addition to the methyl group lowers the aqueous solubility of the halogenated phenols and, perhaps partially as a result of this lower solubility, lowers the activity   For instance, 2,4-dibromothymol and 4,6 dibromocarvacrol (Fig 4) are much less active than 2,4-dichlorophenol and 2,4-dibromophenol

Certain types of enlargement of the central ring nucleus also decrease the aqueous solubility and lower the activity of the dihalo phenols   This may be seen by comparing the results obtained with 2,4-dichloro α naphthol (Fig 3) and 5,7 dibromo 8 hydroxyquino-

line (Fig 4) with the results obtained with 2,4-dichloro and 2,4-dibromophenol

Second, with a fixed arrangement of halogen and alkyl substituents in relation to the phenol group, the dibromo substituted molecule appears to be slightly more effective than the dichloro compound
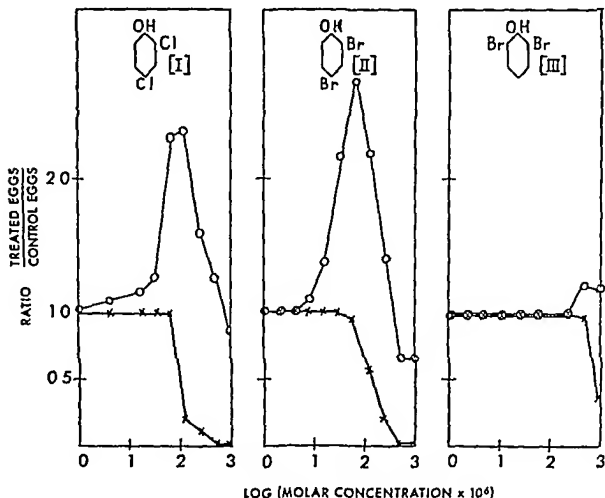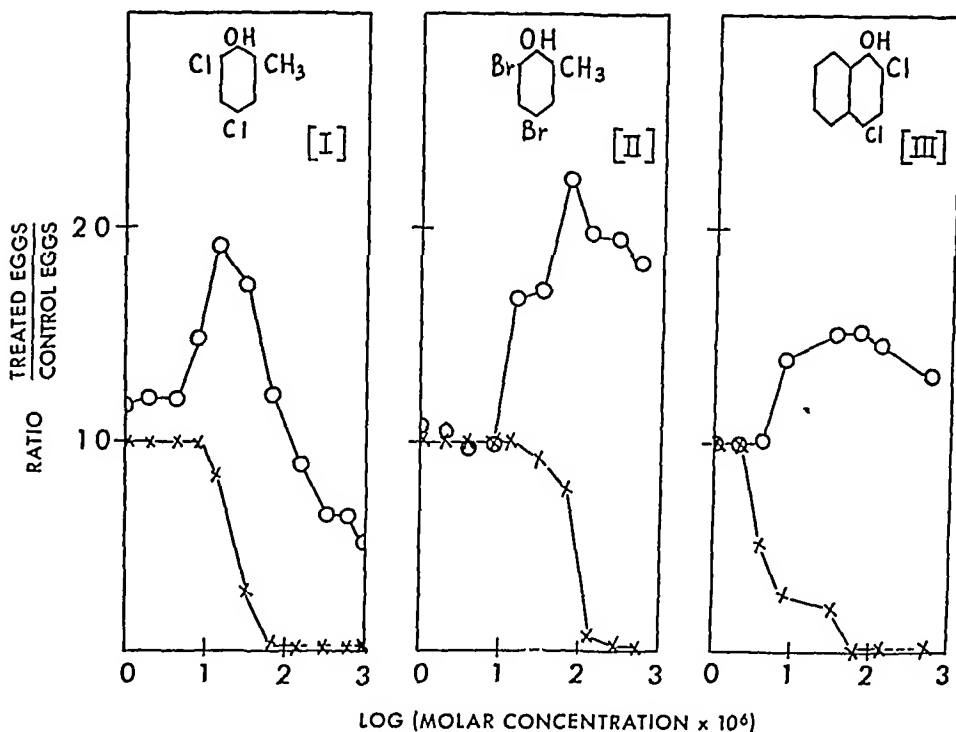

FIG 3 Stimulation of oxygen consumption and block to cell division of fertilized *Arbacia* eggs produced by various concentrations of 4,6-dichloro-*o*-cresol (I), 4,6-dibromo-*o*-cresol (II), and 2,4-dichloro α-naphthol (III) at 20°C The dotted division lines denote irreversible injury Reagent added 15 minutes after fertilization

The phenols, cresols, and thymols may be cited as examples (Figs 2, 3, and 4)

Third, 2,6-dibromophenol is less effective than 2,4-dibromophenol (Fig 2)

Fourth, 2,4-dichloroaniline and the dichloro benzenes, compounds containing respectively an amino and a hydrogen in place of the OH,
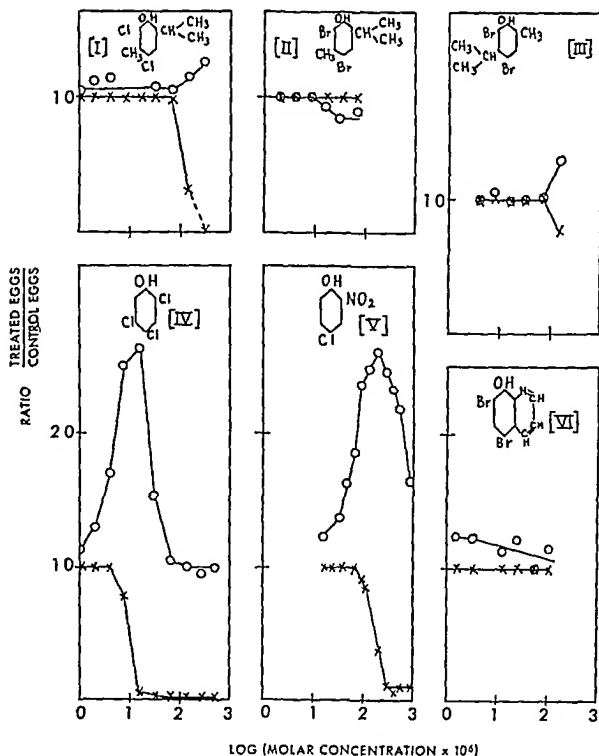
FIG 4 Stimulation of oxygen consumption and block to cell division of fertilized *Arbacia* eggs produced by various concentrations of 2 4-dichlorothymol (I) 2 4-dibromothymol (II) 4 6-dibromocarvacrol (III) 2 4 5 trichlorophenol (IV) *o* nitro-*p*-chlorophenol (V) and 5 7 dibromo 8-hydroxyquinoline (VI) at 20°C Dotted division lines denote irreversible injury Reagents added 15 minutes after fertilization

have no ability to stimulate oxidation or block division in fertilized *Arbacia* eggs

Since *p*-chlorophenol and *p*-chlorothymol were found to possess no respiratory stimulating or reversible division blocking ability, no other monohalophenols were investigated
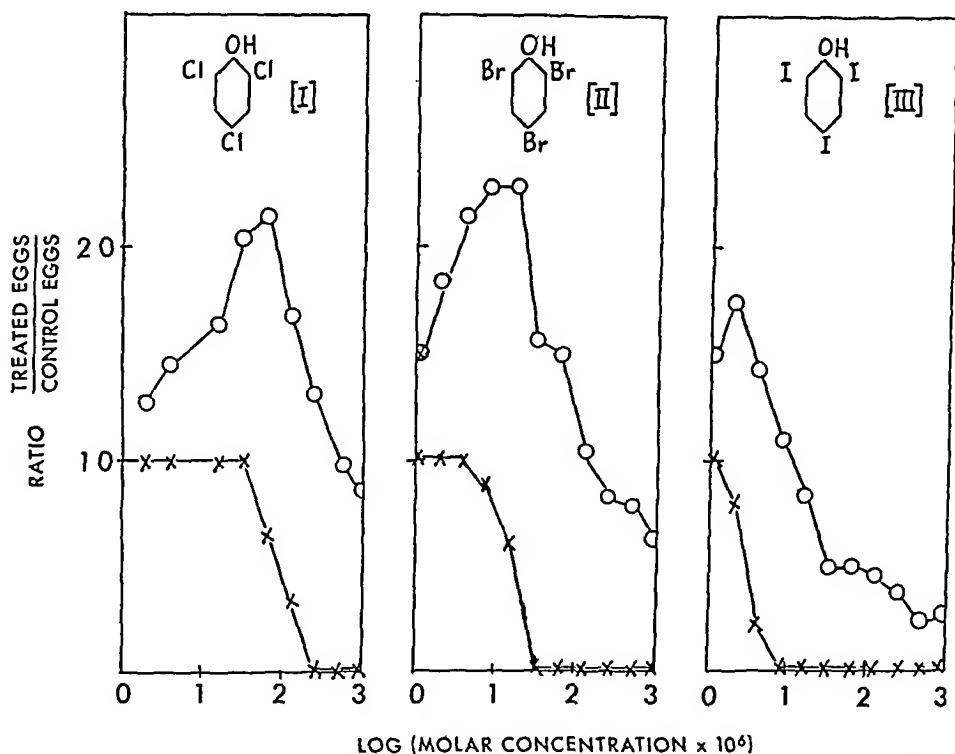
Fig 5 Stimulation of oxygen consumption and block to cell division of fertilized *Arbacia* eggs produced by various concentrations of 2,4,6-trichlorophenol (I), 2,4,6-tribromophenol (II), 2,4,6-triiodophenol (III) at 20°C Dotted division lines denote irreversible injury Reagent added 15 minutes after fertilization

Of the trihalophenols investigated 2,4,5-trichlorophenol gives the highest percentage rise in oxygen consumption It also produces a reversible block to cell division at very great dilution According to both of these criteria, 2,4,5-trichlorophenol compares favorably with any of the nitrophenols (Fig 4)

The 2,4,6-trichlorophenol produces a smaller optimum percentage rise in oxygen consumption and, in order to produce this rise and to

block cell division, requires a higher concentration than its 2,4,5
isomer (Fig 5) The 2,4,6-tribromophenol and 2,4,6 truodophenol
yield a respiratory stimulation and cell division block in smaller con
centrations than those required for the two trichlorophenols first
mentioned, but with the iodo compound the increase in oxygen con-
sumption is small (Fig 5)
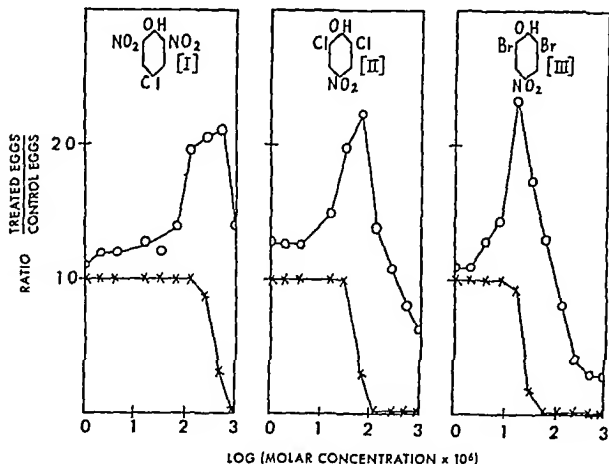
Fig 6 Stimulation of oxygen consumption and block to cell division of fer
tilized *Arbacia* eggs produced by various concentrations of 2 6-dinitro-4-chloro
phenol (I), 2 6-dichloro-4-nitrophenol (II), 2,6-dibromo-4-nitrophenol (III) at
20°C Reagents added 25 minutes after fertilization

High concentration of 2,4,6 tribromo and 2,4,6 truodophenol,
and, to a lesser extent, of 2,4-dibromophenol and 2,4-dichloro α
naphthol produces some cytolysis of fertilized *Arbacia* eggs For this
reason, eggs blocked in division by high concentration of these agents
show a relatively low percentage of recovery when returned to sea
water

In general, phenols which contain both nitro and halo substituents

have respiratory stimulating and division blocking properties very similar to those of the phenols containing only nitro groups and those of the phenols containing only halo groups   An example having one nitro and one chloro group is *o*-nitro-*p*-chlorophenol   This compound (Fig 4) is much more active than either *o*-nitro or *p*-chlorophenol, somewhat more active than 2,4-dichlorophenol, almost equal in activity to *p*-nitrophenol, and slightly less active than 2,4-dinitrophenol

A phenol containing two nitro substituents and one halogen, or a phenol containing two halogen substituents and one nitro has nearly the same respiratory stimulating and division blocking effects as the trihalophenols (Fig 6)

For comparison, the optimum levels of respiratory stimulation produced in fertilized *Arbacia* eggs by representative nitro and halophenols may be expressed in the following order as percentages of normal *p*-nitrophenol, 342, 4,6-dinitro-*o*-cresol, 300, 2,4-dinitrophenol, 292, 2,4,5-trichlorophenol, 264, 2,4-dichlorophenol, 236, 2,6-dichloro-4-nitrophenol, 232, 2,4,6-tribromophenol, 227, 2,6-dibromo-4-nitrophenol, 222, 2,4,6-trichlorophenol, 214, 2,6-dinitro-4-chlorophenol, 205, 2,4,6-triiodophenol, 175, 2,4,6-trinitrophenol, none

The molar concentrations of these reagents required to produce the optimum respiratory rise and initial division block fall in the following order 2,4,6-triiodophenol, 2×10^{-6}, 4,6-dinitro-*o*-cresol, 8×10^{-6}, 2,4,6-tribromophenol, 10^{-5}, 2,4,5-trichlorophenol, 2×10^{-5}, 2,6-dibromo-4-nitrophenol, 2×10^{-5}, 2,4-dinitrophenol, 3×10^{-5}, 2,4,6-trichlorophenol, 6×10^{-5}, 2,6-dichloro-4-nitrophenol, 6×10^{-5}, *p*-nitrophenol, 10^{-4}, 2,4-dichlorophenol, 10^{-4}, 2,6-dinitro-4-chlorophenol, 3×10^{-4} Using these values as a key, other relationships may be found by referring to the figures in this and a previous paper (4)

In having a high division blocking activity associated with a small stimulation of respiration 2,4,6-triiodophenol is similar to 2,4-dinitrothymol

It was obviously desirable to determine whether the dihalo and trihalophenols produced respiratory effects in mammals similar to those described here for single cells   From experiments carried out in

collaboration with Dr K K Chen, it appears that injection or oral administration of 2,4 dichlorophenol, 2,4,5 trichlorophenol, and other halophenols does not produce significant increases in the body temperature or the respiratory rate of rats or dogs

DISCUSSION AND SUMMARY

The dihalo and trihalophenols, and phenols containing both halo and nitro substituents in the same molecule, produce, in fertilized eggs of *Arbacia punctulata*, a rise in rate of oxygen consumption and a reversible block to cell division   To define the conditions which affect the degree of this activity, the following factors have been varied   the arrangement of substituents in the molecule, the concentration of reagent, and the time after fertilization at which the reagent is added

The stimulation of oxygen consumption and reversible block to cell division produced by the dihalophenols are qualitatively the same as those previously produced in fertilized *Arbacia* eggs by certain dinitro phenols   To yield optimum respiratory effect and maximum division block, it usually requires a higher concentration of dihalo than of the corresponding dinitrophenol   For example, with fertilized *Arbacia* eggs at 20°C , 2,4 dinitrophenol, in optimum concentration of 3×10^{-5} molar, raises oxygen consumption to 292 per cent of normal (4) The corresponding values for two dihalo analogues are   2,4 dichloro phenol, 10^{-4} molar and 236 per cent, 2,4 dibromophenol, 6×10^{-5} molar and 282 per cent

The halophenols differ from the nitrophenols in two interesting respects   (a) The monohalophenols produce little or no oxidative stimulation or division block in fertilized *Arbacia* eggs, *p* nitrophenol is very active in both respects   (b) The symmetrical trihalophenols have an appreciable ability to stimulate oxygen consumption and block division, symmetrical trinitrophenol is inactive in both respects (4)

The increases in oxygen consumption produced in fertilized *Arbacia* eggs by 2,4-dichloro and 2,4 dinitrophenol are larger than the per centage increases given by methylene blue and *o* cresol indophenol under the same experimental conditions   The dihalo and dinitro phenols produce a reversible block to the cell division of fertilized

marine eggs   The oxidation-reduction indicators, in contrast to the dihalo and dinitrophenols, block cell division irreversibly and fertilized eggs of *Arbacia* do not recover from optimum respiratory stimulating concentrations of these oxidation-reduction dyes

The present experiments with halophenols are in harmony with and lend considerable support to the hypothesis (4) that nitro and similarly substituted phenols derive their biological activity from the presence and properties of the phenolic OH group, as modified by proper substitution in the phenolic benzene ring

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INTENSITY DISCRIMINATION IN THE HUMAN EYE

I. THE RELATION OF $\Delta I/I$ TO INTENSITY

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I

INTRODUCTION

A uniformly illuminated visual field becomes inhomogeneous in appearance when the light intensity of a part of it is sufficiently changed. The just perceptible difference in intensity ΔI depends primarily on the intensity I secondarily, it is determined by many other variables as well. Because of the relation of ΔI to I, it is customary to use the Weber fraction $\Delta I/I$ as a measure of contrast sensibility. The present investigation is concerned first with the relationship between $\Delta I/I$ and I, and second, with the dependence of this relationship on the area of the test field.

Although the systematic variation of $\Delta I/I$ with I has been frequently determined (Aubert 1865, Helmholtz, 1866, Koenig and Brodhun, 1888, 1889, Blanchard, 1918, Lowry, 1931) the data of different investigators differ in important details, as for example, in the behavior of $\Delta I/I$ at high intensities. In view of the theoretical importance of this function for the photoreceptor process (Hecht, 1935), it is desirable to establish the function definitively, under unambiguous experimental conditions.

The effect of the area of the test field on the capacity to discriminate intensities has been previously studied only at single fixed brightnesses (Lasareff, 1911, Heinz and Lippay, 1928) or over a narrow range of intensities (Cobb and Moss, 1928). The existence of this dependence implies a definite relationship between the intensity difference threshold and the total number of visual receptors stimulated. Its

precise form, and its variation over a wide range of intensities is therefore important for theoretical analyses of the statistical aspects of sensory functions (Hecht, 1928)   By exploring the entire course of the function relating $\Delta I/I$ and I for each of a series of test-fields of widely different areas, it was possible to attain both objectives of the present research simultaneously

II

Apparatus

Two different forms of apparatus have been used   Both presented the same aspect to the subject—a test-field of variable size divided either centrally or otherwise into two parts and surrounded by a large peripheral field   The instruments provided independent control of both parts of the test-field and of the surround over wide ranges of intensity of illumination

Apparatus A —The first arrangement is shown schematically in side view in Fig 1*A*   Light from a 250 watt projection Mazda lamp L, regulated at 2 20 amperes, after passing through a condenser C, two ground-glass screens S, and a biprism, illuminates two rectangular windows w_1 and w_2 A neutral gelatin wedge and balancer WG (perpendicular to the plane of the figure) covers w_2 Over w_1 a neutral filter F matches a point near the thick end of the wedge   The second biprism B and lens L direct the light in such a way that light from w_1 reaches the eye at the artificial pupil P only through the lower half of the biprism, while light from w_2 reaches it only through the upper half   The apical edge of the biprism furnishes a dividing line which is invisible when the fields are matched in hue and brightness   The exit pupil P is a circular opening of 2 0 mm   diameter   It serves to fix the position of the observer's eye and to eliminate the effect of variations in the natural pupil   Wratten neutral filters F in front of the exit pupil control the brightness in discrete steps

Diaphragms D_1 of various apertures placed beyond the lens control the size of the bipartite field D_2 is a larger diaphragm with an outside diameter of 20° and a fixed aperture of 5°37', slightly less than the full aperture of L Diaphragms D_2 and D_1 are covered with a layer of MgO   The surfaces of D_1 and D_2 furnish the surrounding field, they are illuminated by a 500 watt projection lamp LS fitted with condensers, wide aperture projection lens, and mirror M Since the light intensity is controlled by filters at the observer's eye, the surround illumination bears a constant ratio to that of the test-field   In most experiments this ratio was 0 14, and will be called the standard surround brightness

The wedge at w_2, controlled by the observer with a rack and pinion, governs the brightness of the variable half of the test-field   It is 15 cm long, and its position can be read to 0 1 mm on an attached scale   It was calibrated with a vacuum thermocouple and a Leeds and Northrup HS galvanometer, infrared radiation being excluded with a solution of copper sulfate   Empirical justification for this
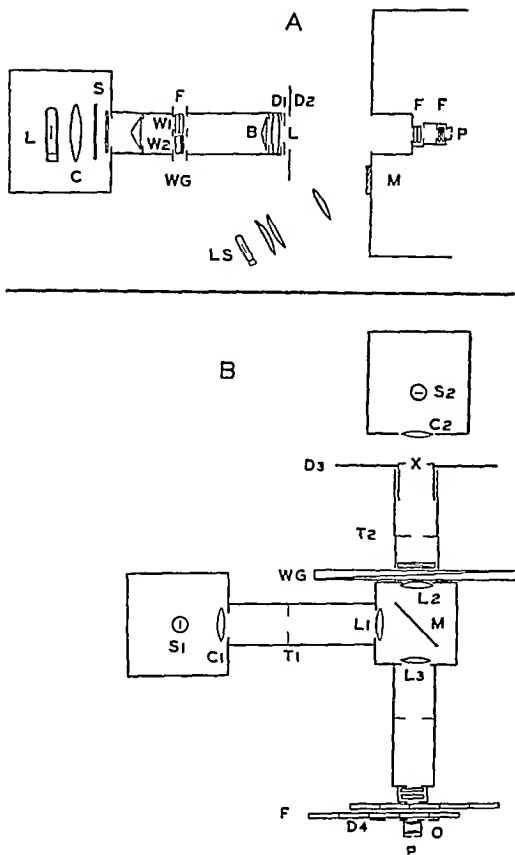
Fig 1 A diagrammatic side view of the optical arrangements used in the measurement of intensity discrimination with Apparatus A is shown in the upper portion marked A, while the arrangements of Apparatus B are shown in top view in B

method was obtained with decimal neutral filters which had been calibrated visually with a Martens polarization photometer   Within the error of the determinations, the logarithm of the wedge transmission was a linear function of the scale readings   Wedge and balancer together covered a total range of transmissions of approximately 2 5 to 1

The decimal filters in front of the pupil are mounted in two movable frames, each containing five squares transmitting, respectively, approximately 1, 1/10, 1/100, 1/1000, and 1/10,000 of the incident light   Two filters, one from each of the two frames, are in front of the observer's eye simultaneously   An additional filter transmitting either 1/2 or 1/4 can be inserted, thus making possible 27 different light intensities over a range of about 1 to 400,000,000   The filters were calibrated with a Martens polarization photometer, using the method described by Hecht, Shlaer, and Verrijp (1933)   These authors have also described the method used for measuring the absolute brightness of the field   The highest brightness was close to 16,000 millilamberts   To convert this to photons, that is, to units of retinal illumination, it is multiplied by $10r^2$ where r is the radius of the exit pupil in millimeters   Here $r = 1$, and the highest intensity therefore is about 160,000 photons

To translate the wedge readings into $\Delta I/I$, the wedge-setting at which both halves of the field have the same brightness must be known   This matchpoint which changed with the age of the apparatus, was determined for every experiment, the average of at least 10 settings being taken as the day's value   Errors in this value may affect the smaller ratios of $\Delta I/I$ very considerably

The apparatus described above proved inadequate in several important respects   The maximum intensity was too low, the test-field was too rigid in pattern, small errors in matchpoint determination weighed heavily in the computations, and, above all, the two halves of the field showed hue differences which were impossible to exclude, and which, though imperceptible at low illuminations, were a source of annoyance and uncertainty at high intensities   Apparatus B was constructed to circumvent these difficulties

Apparatus B —In this apparatus one light of intensity I illuminates the entire visual field, while another light ΔI adds its illumination only to that portion of the field which constitutes the test-spot   Thus, small hue differences between the two lights are diluted and disappear   Higher illuminations are achieved by an optical system in which diffusion screens are unnecessary and have been omitted

In Fig 1B the lamps S_1 and S_2 are 100 watt concentrated-filament projection Mazdas, operated at 0 86 amperes   The relative brightness of the lamps is followed by measurement of the wedge matchpoint as described below, changes in absolute brightness are measured by comparison with a standard lamp, using a Weston photronic cell screened by a solution of copper sulfate   No significant change was noted in the duration of the experiments

An enlarged image of the filament S_1 is produced by the condenser C_1 on the achromatic lens L_1 A slightly enlarged image of the diaphragm at C_1 is produced, by L_1 and L_3 and the half-platinized mirror M, at the focus of the 10 × Ramsden

ocular O The observer, looking through the 3 mm artificial pupil P sees an en larged, intensely illuminated image of the aperture of C_1 If the apparatus is carefully aligned, this illumination is critically uniform   The field size is con trolled by diaphragms cut in a metal slide D_4 at the focus of the ocular

The optics of the beam from S_2 are similar to those of S_1 except that the size and shape of its image in the field are regulated by the diaphragm λ, which is so mounted that it may be rotated manually or with a motor, by the attached disc D_3

The wedge WG in the path of the light from S_2 covers a transmission interval of about 1 to 50   When its dense end covers the lens, the light added to the large field illuminated by S_1 is too small to be perceptible   When its clear end is before the wedge, the added light ΔI is about 60 per cent of I Values of $\Delta I/I$ up to about 0 60 are therefore directly measurable   Higher values are obtained by re- ducing I relative to ΔI with a neutral filter (transmitting 1/2) inserted between the mirror and L_1 Similarly, values of $\Delta I/I$ smaller than about 0 012 are obtained by inserting the filter between the mirror and L_2

The wedge was calibrated by two different methods, without removal from the apparatus   The first time a Weston photronic cell, screened from infrared and ultraviolet light, was connected to a sensitive low resistance millivoltmeter pro- vided with a shunting arrangement that gave different ranges of sensitivity with a constant total circuit resistance   6 months later, a visual calibration was made with a Macbeth illuminometer   Both methods showed a linear relation between the logarithm of the transmission and wedge scale readings   the slopes agreeing within 2 per cent   The slope obtained visually has been used in most of the computations

The filters in front of the ocular control the intensity as in Apparatus A and were similarly calibrated   Experiments with *monochromatic*" filters were per- formed by inserting Wratten filters in the space between the mirror M and the lens L_3 where they intercept light from both beams

It is not possible to measure the apparent brightness of the visual field as directly as in the case of Apparatus A   owing to the use of a short focus ocular However, an approximately correct value was obtained binocularly, both eyes being fitted with artificial pupils   The maximum apparent brightness, with all filters removed   was about 2,000 000 photons

When a fixation point was required, a neutral filter transmitting 1/4 of the light and having a very fine hole punctured through it was placed at D_4 The result was a shift of 0 60 log units in the intensity scale and the production of a small bright spot in any desired part of the field

To calculate $\Delta I/I$ it is necessary to determine the wedge setting at which the brightnesses of S_1 and S_2 have some known ratio   This was done by careful dia- phragming at C_1 and at λ to obtain a divided field each half of which received light from one of the two beams only   With a neutral filter of known transmission inserted at L_1 the wedge was then moved until a match was obtained

One advantage of the present apparatus is that the per cent error in $\Delta I/I$ caused by error in setting the wedge is the same at all wedge positions   likewise small errors

in estimating the matchpoint will affect the accuracy of all values of $\Delta I/I$ by the same factor   Another advantage is that it provides a flexible test-field pattern, in this way it can be set so that the recognition of a pattern is required of the observer, here the lack of a physical counterpart to the dividing line increases the definiteness of this requirement   Against these advantages must be noted the aberrations produced by the ocular, noticeable with extremely small test-spots, and the difficulty of providing a large surround when a bipartite field is used

III

Procedure

Before each experiment, the observer was dark-adapted for 25 minutes, occasionally, when using small test-areas falling within the fovea, shorter periods were allowed, special experiments having shown that $\Delta I/I$ did not change after 6 minutes dark adaptation, when small fields were used   Measurements were always begun at the lowest intensities   Before beginning a setting, the observer, protected from all stray light, allowed about a minute for adaptation to the prevailing intensity, at very high intensities, 5 minutes were sometimes allowed   The setting occupied 3 minutes or longer and consisted in gradually adding light to one part of the field until the difference in intensity became perceptible

The magnitude of $\Delta I/I$ is affected by the procedure and by the criterion of certainty adopted   In this paper the criterion was the appearance of a distinct boundary between the fields   With the first apparatus the fields were stationary, with the second apparatus the test-field was rotated slowly at constant speed (10 R P M), and the observer was required to name the direction of rotation   The latter procedure gave reproducible averages with two or three settings, and made it possible to measure all the intensities in a single day   Each experiment made in this way was repeated at least once   Curves obtained on different days vary somewhat, but the differences are consistent throughout   With the first apparatus, the entire intensity interval could be covered in one day only by measuring at fewer (alternate) intensities, points in between were then determined on subsequent days   Four such complete sets were made for each size of field   This method is slow, moreover, averaging different days' work tends to obscure certain discontinuities in the data   However, the averaged data are more independent of the time element, and comparisons of results with different areas may be made with confidence

An assistant, Miss Dorothy Pope, controlled the current in the lamps, and read and recorded the wedge settings   With the second instrument, she also changed the position or direction of rotation of the test-area   The author is greatly indebted to Mr Simon Shlaer of the Biophysics Laboratory for practical advice and assistance in the design and construction of the two instruments described in the preceding section

IV

Results of the Measurements

(A) Main Data

During 3 years, over 6000 measurements of $\Delta I/I$ have been made on the right eye of the author at intensities between 0 02 and 2,000,000

TABLE I

Relation of $\Delta I/I$ to log I for different size of field, standard surround present All data represent averages of 10–20 determinations on 4 days, except the two fields 3 44' and 16 which were obtained each on a single day

Log I in photons	ΔI/I				Log I in photons	ΔI/I						
	5 36	4 40	3 44	2 14		56	41	31	23 5	16 1	12 2	9 1
5 212	0 014	0 014	0 011	0 015	4 991	0 027	0 054	0 043	0 053		0 144	0 525
4 892	0 012	0 013	0 009	0 015	4 671	0 023	0 040	0 041	0 030	100	0 123	0 252
4 595	0 015	0 010	0 009	0 014	4 374	0 028	0 045	0 045	0 046		0 127	0 315
4 256	0 014	0 016	0 016	0 020	4 035	0 025	0 055	0 045	0 050		0 139	0 304
3 963	0 016	0 015	0 015	0 021	3 715	0 025	0 045	0 048	0 045	157	0 166	0 331
3 639	0 016	0 015	0 014	0 017	3 418	0 028	0 052	0 048	0 060		0 161	0 471
3 175	0 015	0 012	0 014	0 019	2 954	0 028	0 061	0 055	0 072		0 178	0 499
2 855	0 015	0 017	0 013	0 020	2 634	0 034	0 056	0 058	0 066	144	0 190	0 452
2 558	0 019	0 013	0 014	0 023	2 337	0 037	0 063	0 061	0 080		0 185	0 524
2 168	0 015	0 018	0 016	0 027	1 947	0 045	0 071	0 072	0 105		0 211	0 498
1 848	0 019	0 020	0 017	0 032	1 677	0 054	0 068	0 085	0 109	270	0 274	0 465
1 551	0 023	0 021	0 075	0 038	1 330	0 070	0 102	0 099	0 147		0 294	0 549
1 124	0 028	0 031	0 036	0 056	0 903	0 095	0 127	0 178	0 315		0 388	0 690
0 804	0 036	0 042	0 037	0 0/6	0 583	0 139	0 218	0 236	0 405	498	0 620	0 813
0 507	0 055	0 047	0 057	0 112	0 286	0 242	0 304	0 379	0 579			
0 168	0 089	0 098	0 100	0 168	-0 053	0 322	0 540	0 621				
-0 152	0 141	0 163	0 180	0 306	-0 373	0 587						
-0 449	0 221	0 243	0 313	0 521								
-0 896	0 313	0 400	0 462	0 854								
-1 216	0 408	0 452	0 594									
-1 513	0 444	0 540	0 664									
-1 888	0 577	0 766	0 858									
-2 208	0 764											

photons, using test fields with diameters between 24°4' and 9'   In this communication the chief concern will be with the relation of $\Delta I/I$ to the intensity, though the results can hardly be presented without describing the more general aspects of the area effects

The main data are in Tables I, II, and III   Those in Table I were

obtained with a surround of constant size (Apparatus A) kept at an illumination approximately 1/7 that of the test-field Those in Table II were obtained under similar conditions but with no surround Table III (Apparatus B) deals with large fields and presents the data of individual experiments singly Because of the large fields, no surround was necessary, as will be shown later Fig 2 represents a

TABLE II

Relation of $\Delta I/I$ to Log I for Different Size of Field, No Illuminated Surround Average of 15–20 Readings on 4 Days

Log I in photons	$\Delta I/I$ 4°40′	Log I in photons	$\Delta I/I$				
			56′	41′	31′	23 5′	16 1′
5 212	0 014	4 991	0 041	0 049	0 058	0 168	0 588
4 892	0 011	4 671	0 030	0 034	0 065	0 128	0 383
4 595	0 013	4 374	0 031	0 041	0 065	0 135	0 373
4 256	0 017	4 035	0 027	0 044	0 060	0 149	0 438
3 936	0 016	3 715	0 031	0 047	0 079	0 160	0 388
3 639	0 015	3 418	0 030	0 046	0 072	0 143	0 340
3 175	0 015	2 954	0 040	0 053	0 075	0 168	0 312
2 855	0 016	2 634	0 033	0 055	0 088	0 158	0 259
2 558	0 017	2 337	0 041	0 061	0 089	0 158	0 248
2 168	0 021	1 947	0 054	0 071	0 090	0 165	0 233
1 848	0 022	1 627	0 050	0 085	0 112	0 182	0 239
1 551	0 029	1 330	0 066	0 105	0 136	0 200	0 282
1 124	0 038	0 903	0 109	0 150	0 185	0 289	0 377
0 804	0 050	0 583	0 138	0 210	0 297	0 372	0 588
0 507	0 066	0 286	0 247	0 380	0 469	0 671	0 928
0 168	0 126	−0 053	0 393	0 624	0 860		
−0 152	0 183	−0 373	0 604				
−0 449	0 284	−0 670	1 211				
−0 896	0 475	−1 117					
−1 216	0 507	−1 437					
−1 513	0 660	−1 734					
−1 987	0 821	−2 208					

portion of these data, a selection having been made to avoid crowding The two largest fields are from Table III (two sets of data for the largest field are shown), the others from Table I It is apparent that the measurements though made at different times, with different apparatus, and different light sources, are homogeneous and represent essentially the same phenomena This is best shown by the

TABLE III

Relation of $\Delta I/I$ to Log I for Large Slowly Rotating Fields   The Columns Represent Specimen Averages of Readings Made on Single Days

Under each "Log I in photons" column the heading $\Delta I/I$ spans the two field columns (24 or 17) and the column 5 38.

Log I in photons	24	24	5 38	Log I in photons	17	17	5 38
6 300		0 0169					
5 980			0 0202				
5 643		0 0163		5 707		0 0174	0 0276
5 345			0 0231	5 409	0 0174	0 0155	
5 024		0 0158		5 088	0 0176	0 0154	
4 677			0 0202	4 741	0 0166	0 0161	0 0272
4 264		0 0136		4 328	0 0169	0 0143	
3 943			0 0205	4 007	0 0173	0 0139	0 0206
3 606		0 0147		3 670	0 0168	0 0160	
3 257	0 0135		0 0196	3 321	0 0172	0 0140	0 0226
2 937		0 0127		3 001	0 0160	0 0130	
2 599	0 0137		0 157	2 663	0 0160	0 0125	0 0211
2 213		0 0154		2 277	0 0168	0 0155	
1 893	0 0164		0 0192	1 957	0 0197	0 0159	0 0282
1 555	0 0185	0 0214	0 0207	1 619	0 0217	0 0189	
1 257	0 0232	0 0246	0 0782	1 619	0 0215		
0 936		0 0285	0 0318	1 321	0 0247	0 0218	0 0347
0 623		0 0422		1 000	0 0352	0 0344	0 0434
0 599	0 0396	0 0423	0 0547	0 687			0 0528
0 286		0 0548		0 663	0 0447	0 0484	
0 193	0 0552		0 0928	0 350			0 0910
−0 120	0 0780	0 0893	0 157	0 257	0 0666	0 0779	
−0 120			0 151	−0 184			0 168
−0 128		0 0875		−0 192	0 100	0 113	
−0 441	0 106	0 114	0 227	−0 505			0 316
−0 441	0 114			−0 529	0 134	0 151	
−0 465		0 120		−0 529		0 145	
−0 465				−0 842			0 477
−0 778	0 162	0 150	0 343	−0 864	0 174	0 203	
−0 799		0 162		−1 176			0 556
−1 112	0 189	0 179	0 463	−1 183	0 195	0 227	
−1 119		0 177		−1 496			0 702
−1 432	0 197	0 202	0 536	−1 521	0 222	0 253	
−1 457		0 218		−1 834			
−1 770	0 240	0 244		−1 930	0 294	0 324	
−1 866		0 281		−2 243			
−2 179	0 331	0 339		−2 250	0 469	0 407	
−2 186		0 317		−2 563			
−2 499	0 419			−2 587	0 542		
−2 523			0 711	−2 900			1 188
−2 836	0 702		0 756				

curves drawn through them, these have all been traced from the same stencil, and are the theoretical curves derived by Hecht (1934, 1935) for the general relation between $\Delta I/I$ and I　Their applicability will be discussed later
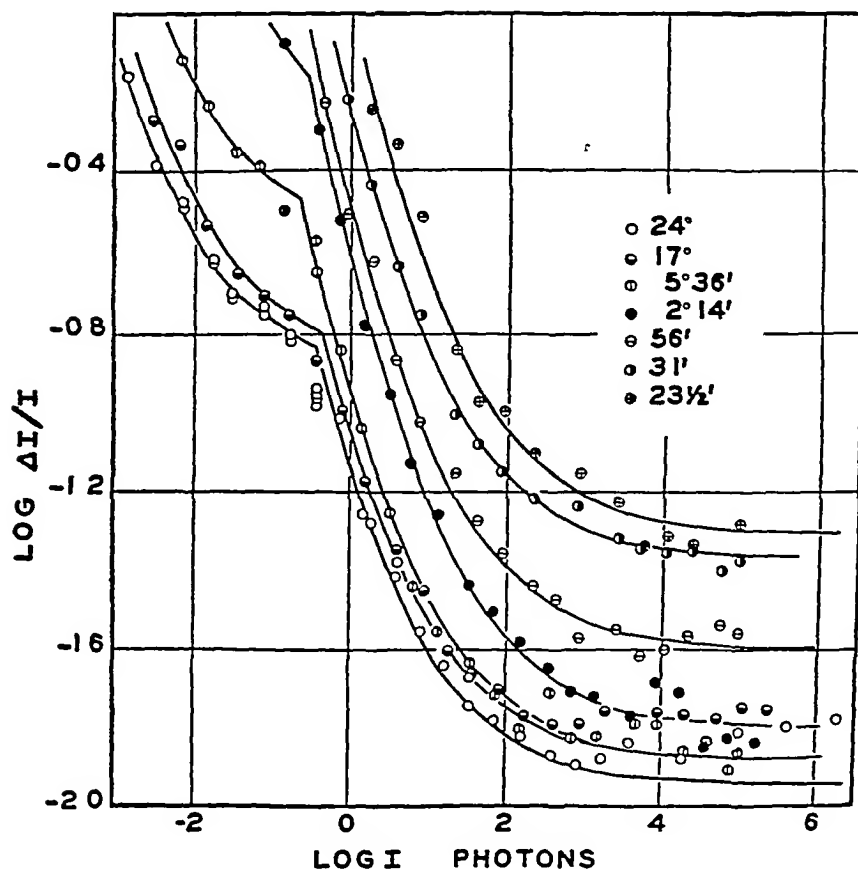
FIG 2　Intensity discrimination in the human eye as influenced by illumination and size of field　The data for all except the two largest fields are taken from Table I and represent averages of 15 or 20 readings on four different days　The data for the two largest fields are taken from Table III and represent averages of 3 to 5 readings in single experiments　All the curves are identical in form, merely shifted in position on the ordinates

Several characteristics of the data in Fig 2 require particular attention　In confirmation of Aubert, of Koenig and Brodhun, and of Blanchard, $\Delta I/I$ is large at low intensities, and diminishes rapidly

as I is increased   The decrease becomes more gradual at high values
of I until there is practically no further change beyond about 1000
photons   Above this intensity, the points scatter noticeably cor
responding to increased variability of the individual readings, and to
greater subjective difficulty with criteria   With the two largest
fields there are apparently systematic discrepancies in this high
intensity interval, the data lying above the curve   The individual
experiments with these fields show this deviation, but its extent varies
They were all made with Apparatus B and may represent an experi-
mental artefact   The largest field of Table I (Apparatus A) is the
same size as the smallest of Table III (Apparatus B), and it does not
show this discrepancy [1]   In all but these very large fields, $\Delta I/I$ be
comes practically constant at the highest intensities, there is conse
quently no optimum intensity for discrimination

   These data are in disagreement with the findings of Koenig and
Brodhun (1889) in a number of ways   In particular, these investiga-
tors record a systematic and substantial increase in $\Delta I/I$ at high
intensities   The secondary rise in $\Delta I/I$ has already been questioned
by Guild (1932), and is not confirmed by the careful measurements
of Lowry (1931) who reports an almost insignificant rise of doubtful
validity (smaller even than shown by the data of Table III)   Lowry's
high intensities and our own are easily as high as and very likely
higher than those achieved by Koenig and Brodhun   Their rise
may depend on incomplete light adaptation (cf Hecht, 1935 and
experiments on the effect of a surround, below)   Neither Wolf's
measurements with the honey bee (1933a,b) nor the data of Hecht
and Wald (1934) on *Drosophila* show an upturn of $\Delta I/I$ at high in
tensities   Measurements with *Mya arenaria* (Hecht, 1924, 1935)
show minima in some experiments, but these depend on single high
points only, and are therefore uncertain

   At low intensities the curves approach a limiting straight line with
a slope of -1 This means that ΔI becomes constant, independent
of I Thus, although Weber's generalization $\Delta I/I = k$ is true as a

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[1] The rise at high intensities in the data for *very small* fields in Table I is shown
below to be the result of incomplete retinal adaptation

limiting condition at one end of the curve, at the other end the contrary limit is attained, and $\Delta I = k$ [2]

(B) Area

Discrimination is better in large fields than in small ones   The entire relation between $\Delta I/I$ and I is displaced with area   The displacement is principally upward, but there is probably also a slight shift along the abscissa   This is contrary to what Wolf (1933b) found with the honey-bee by the moving stripe method   There the displacement of the data caused by reducing the stripe width was mainly horizontal, toward higher intensities   Careful replotting of his data (Hecht, 1935) shows some vertical displacement also

The data for the different areas fall sharply into two groups   Those below 2° are continuous and are best represented by one curve   Those above 2° show an obvious discontinuity and are best represented by two curves   The transition occurs at about 0 3 photons, and is responsible for the low values of $\Delta I/I$ which are obtained with large fields at intensities far below those at which discriminating capacity practically vanishes in smaller areas   The smaller the test-field the higher the level on the lower curve at which the break occurs   Slight changes in the position of the break occur in individual experiments with the same size field, the two curves which intersect at the break may thus be affected independently in day-to-day variations, and hence probably correspond to distinct visual processes

As Hecht (1935) has shown, this break is clearly present in the original data of Aubert (1865), and in the measurements of Blanchard (1918)   Neither Koenig and Brodhun's data with white, blue, and violet light, nor the rather sparse measurements by Holladay (1926) with white light show the presence of a break, but it is strikingly present in the orange, yellow, and green (Brodhun's eye) light data of Koenig and Brodhun (1888)   It is probably significant that all previous data which show a break are easily fitted with the theoretical curves drawn in Fig 2 (cf Hecht, 1935), whereas the others are not

[2] Fechner's modification of the Weber law (Fechner, 1860), $\Delta I/(I + C) = k$ possesses these two limits, and is in fact algebraically identical with another of Hecht's theoretical equations which describes intensity discrimination in *Drosophila*

In the absence of a break, the points in that region are lower, and act as if they represent a summation of the two component curves This is equivalent to saying that even the steep descending portion of these data cannot be described by Hecht's equation Thus the data of Koenig and Brodhun stand apart from the data of all other careful investigators on three major counts

Numerous studies of human visual phenomena have demonstrated the general applicability of von Kries' Duplicity theory (1929) The relations between visual acuity and intensity (Hecht, 1928), between critical fusion frequency of flicker and intensity (Hecht and Verrijp, 1933a, b), and between threshold and time of dark adaptation (Kohlrausch, 1931, Hecht, Haig, and Wald, 1935) show two parts corresponding to the two parts of von Kries' dual mechanism, the rods and cones The various changes in the relation between $\Delta I/I$ and I brought about by differences in experimental procedure are entirely consonant with von Kries' hypothesis of the separate functions and sensibilities of the retinal rods and cones [3]

(C) Color

Two methods of differentiating rod and cone function (Hecht, 1921, Kohlrausch, 1931, Hecht and Verrijp, 1933a) are (1) to alter the spectral composition of the stimulating light, and (2) to control the position of the test image on the retina The first depends on the fact that different regions of the spectrum have widely different stimulating efficiencies for the rods and cones Light from the red end of the spectrum is especially useful because it gives a maximum ratio of cone to rod sensibility The second method depends on the fact that the center of the human retina is practically rod free while peripheral regions contain increasingly more rods than cones The

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[3] Certain of the measurements with very large fields obtained with Apparatus B show a persistent elevation above the theoretical curve at high intensities which may possibly mean that a third receptor system takes over discrimination at the highest intensities in the same way that the cones take it over from the rods at more moderate brightnesses Since this involves the assumption that $\Delta I/I$ would otherwise rise at high intensities and since the possible evidence for it both physiological and histological (Rochon Duvigneaud 1907) is dubious, no further consideration will be taken of this possibility here

measurements to be described in this connection are incomplete, but conclusive in their meaning

Wratten filters Nos 70, 88, and 89A were used to isolate the red end of the spectrum, they transmit light of wavelengths longer than 670, 680, and 690 mμ respectively In Fig 3 the measurements with No 70 filter for 24° and 5°38′ fields are compared with typical white light curves for the same areas   The brightness scale for the
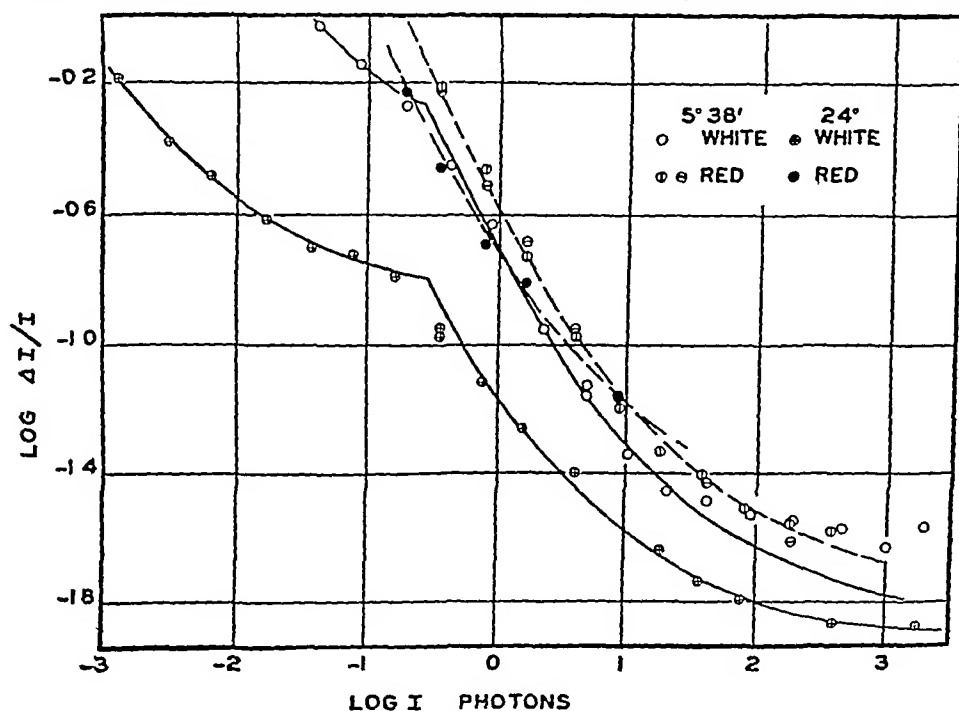
FIG 3 Intensity discrimination as affected by use of red light with two different sizes of test-field For comparison, data obtained with white light for the two fields are also included in the figure The points represent averages in a single experiment White light data for the larger field are not strictly comparable to the others since they were obtained with a somewhat lower criterion of judgment

red and white lights is the same, having been adjusted by means of factors determined in previous flicker measurements with the identical filter (Hecht and Verrijp, 1933a) [4] With the smaller field, the red

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[4] The appropriate factor appears to be a function of the field size Thus, the adjustment for the smaller field results in almost perfect superposition of the lower components, while the same factor does not quite produce this effect with the data for the larger field The correction factor used was actually determined in flicker experiments with a small (2°) field

light data differ strikingly from those with white light by the complete absence of the upper curve There is also a small vertical shift in the curves The red light data fit the standard curve closely, even
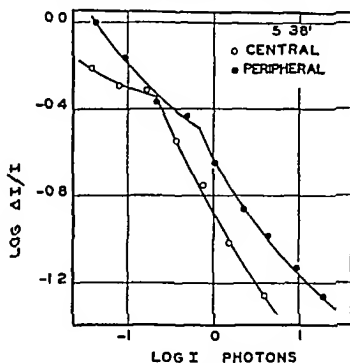
FIG 4 Intensity discrimination in a field of 5 38 as affected by the position of the retinal image The points represent averages obtained in a single experiment The open circles are measurements with central fixation the solid circles measurements with fixation 4 *down*

TABLE IV

The Relation of ΔI/I to Log I for a Field with a Diameter of 5 38 Placed 4 Off Center

Log I in photons	ΔI/I
1 611	0 0547
1 291	0 0729
0 953	0 104
0 655	0 136
0 334	0 224
−0 003	0 366
−0 409	0 427
−0 730	0 690
−1 067	0 986

better than do the data for white light which exhibit slight deviations just below the intersections of the two curves (though these deviations appear only in experiments with Apparatus B, and are hence

of secondary significance)   The differences between the two sets of data obtained with red and white light are very similar to differences brought about by changes in the size of the field
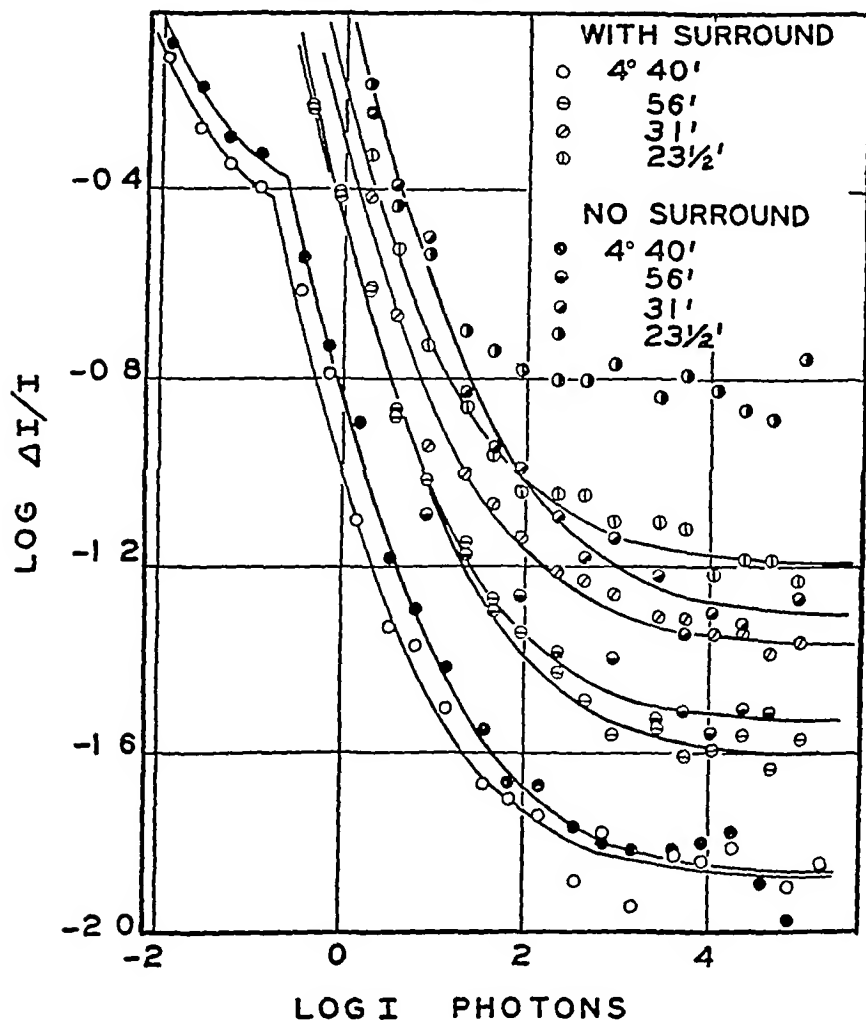
FIG 5   Intensity discrimination in the human eye as affected by the presence of a surround   The surround brightness is approximately 0 14 times the test-field brightness   The data are taken from Tables I and II and represent averages on four different days   The curves are the same as in Fig 1   No curve is drawn through data for 23 5' field obtained without surround

These differences are even more apparent for the 24° field   Here the difference between the two curves is not entirely due to the spectral composition of the stimulus because the white light data were

gotten with a criterion which yielded values of log $\Delta I/I$ about 0 15 units lower than those usually obtained   There remains nevertheless a large vertical displacement   The greatest change is shown at low intensities where the white light data extend almost two log units below the red   The absence of the break and of the low intensity component is obviously due to the low stimulating efficiency of red light for rods   It should be noted, in confirmation of this interpreta
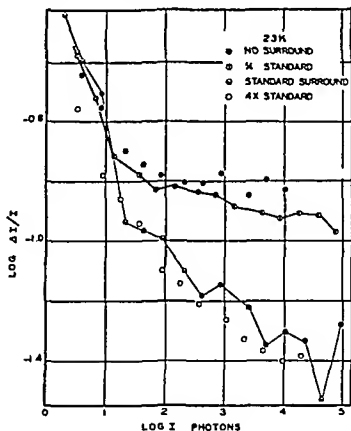
FIG 6 Intensity discrimination data for a field of 23 5' as affected by the brightness of the surround   The lines connecting two of the sets of points have no significance other than aiding the reader to separate the data   The points represent averages obtained on four different days

tion, that there is very little difference between the white light data for the small field and the red light data for the large

These results confirm the conclusions drawn by Hecht (1935) from the red, orange, and yellow light data of Koenig and Brodhun, and already referred to   For 670 mμ their measurements fall on a single curve representing cone function, while for 605, 575, and (for Brod hun's eye) 505 mμ, the low intensity, rod section becomes increasingly larger, as would be expected from the relative sensibilities of the rods

and cones to these different portions of the spectrum   The blue and violet data, like their white data, do not show any break, and may indicate summation of the two functions under particular experimental conditions

(D) Excentric Fields

The method of testing the duplicity theory by varying the retinal position of the test-field helps to distinguish between effects of area due to
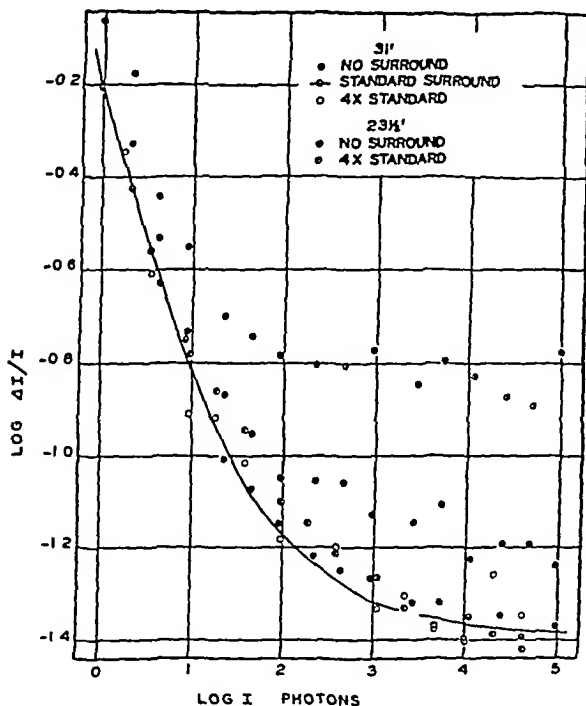
FIG 7 Data for a test-field of 31' as affected by surround brightness   For comparison, measurements obtained with a 23 5' field with no surround and with a strong surround are included   The points represent averages obtained on four different days

size and those due to kinds of retinal element dominant in the different areas   The data show that it is possible to imitate some features of centrally fixated large area data with smaller areas viewed excentrically   Table IV presents measurements with a 5°38' field placed 4° below the center   They are compared in Fig 4 with central fixation data   In the peripheral data the rod curve is displaced downward 0 34 units, and the cone curve upward about the same amount   Sec-

ondarily, there is a horizontal displacement of the rod curve, as well Obviously in the periphery the cones are a less sensitive system than in the center The reverse is true for the rods though the secondary horizontal displacement partly obscures this The position as well as the size of the retinal image determines the nature of the results Measurements by Kravkov (1931) are consistent with this conclusion

TABLE V

The Effect of Surround Brightness on the Relation of $\Delta I/I$ to Log I The Data Represent Averages of 10–20 Determinations on 4 Days

23 5 field				31 field	
Surround 4 times standard		Surround 1/4 of standard		Surround 4 times standard	
Log I in photons	$\Delta I/I$	Log I in photons	$\Delta I/I$	Log I in photons	$\Delta I/I$
4 610	0 0378	5 212	0 145	4 610	0 0453
4 290	0 0412	4 892	0 108	4 290	0 0553
3 993	0 0397	4 595	0 122	3 993	0 0403
3 654	0 0432	4 256	0 124	3 654	0 0421
3 334	0 0470	3 936	0 120	3 334	0 0498
3 037	0 0548	3 639	0 124	3 037	0 0467
2 573	0 0617	3 175	0 131	2 573	0 0634
2 253	0 0739	2 855	0 143	2 253	0 0717
1 952	0 0798	2 554	0 147	1 952	0 0660
1 566	0 115	2 168	0 153	1 566	0 0976
1 246	0 138	1 848	0 150	1 246	0 121
0 949	0 164	1 551	0 167	0 949	0 124
0 522	0 280	1 124	0 193	0 522	0 248
		0 804	0 303	0 202	0 451
		0 507	0 420		
		0 168	0 758		

(E) Surround

The measurements given in Table I were made with the test field surrounded by an area the brightness of which was 0 14 of the test field The absence of such a surround affects the data with small test fields considerably more than those with large fields Data obtained without surround scatter more widely, especially at high intensities Fig 5, constructed from Tables I and II, shows that with the possible exception of the smallest field, absence of a surround does not change the *form* of the relation between the variables The position of the

curves, however, is distinctly shifted upward to an increasing extent as the fields are reduced   With the 31′ field the differences are considerable, while with the 23 5′ field they are so great that merely shifting the curve does not suffice to describe it   There appears also to be a smaller, and much more variable displacement toward higher intensities, the average for all the curves without a surround is about 0 2 log units

Since absence of a surround has so marked an effect in small fields, it is natural to ask whether the surrounds used in obtaining the data of Table I were sufficient   Fig 6 shows the data for a 23 5′ field without a surround, with the standard surround of 0 14 of the test-field, and with 1/4 of and 4 times the brightness of the standard surround   It is apparent that with this small field even a weak surround influences the position of the data   The difference between the standard and 4 times the standard surround is not very large, but is probably real   Fig 7 shows the data for the 31′ field   For this area there is no difference between the standard and 4 times the standard, the effect is therefore maximal   The points in Figs 6 and 7 are from Tables I, II, and V

In Fig 7, data for the 23 5′ field is introduced for comparison   It is apparent that the values of $\Delta I/I$ with the two fields are almost identical when determined with a proper surround   There appears a possible slight horizontal displacement, but no vertical one   These measurements show that below a field size of 30′ diameter wholly erroneous impressions would result from comparing measurements with fields of various sizes without a surround of the same order of brightness as the test-field intensity   For this reason areas smaller than this were not included in Fig 2 although given in Table I

V

Interpretation

The data have shown that visual intensity discrimination may be regarded as a function of two distinct groups of receptors which differ in retinal distribution, in spectral sensibility, and in intensity threshold   The group predominating at low intensities is more peripherally distributed and has its maximum spectral sensibility further to the blue than the group which discriminates at high intensities   These

three differences, among others, identify the former as rods, the latter as cones

Because of the break in the data for all fields larger than 2° it is supposed that these two types of receptors function practically independently of each other over their distinctive intensity ranges   This supposition is strengthened by the fact that the data for fields below 2°, the data for all fields with red light, and the large field data to the right of the break are all described by the same curve   Moreover, the effects of area, surround brightness, wavelength, and fixation can all be described on the basis of these particular curves which change in position but not in form

It is possible that this independence of rod and cone function is not absolute, and that due to a limited kind of summation under certain conditions, the capacity to discriminate is better than it would be if either of the two systems were functioning alone   This appears to occur, if at all, only at intensities near the break and only for the largest fields used, with Apparatus B   Summation may be invoked to explain the absence of a break in the blue and violet data of Koenig and Brodhun, yet the fact that the break is also absent in their white data, whereas our own white data and those of Aubert and of Blanchard show the break strikingly (Hecht, 1935) speaks against such an interpretation   Nevertheless certain aspects of the quantitative relation between $\Delta I/I$ and area, to be reported in another communication, tend to support the idea of a limited, additive effect under certain conditions

The curves drawn through the data in all of the figures are theoretical ones and are taken from the equations recently derived by Hecht (1934, 1935) as a basis for the intensity discrimination of a variety of photosensory systems   This assumes that in order to discriminate between two intensities I and $I + \Delta I$ there must be produced a constant difference in the rate of photochemical decomposition at the moment when the receptor is exposed to the just perceptible increment ΔI after adaptation to I If the first step in the photosensory process is assumed to be a cyclical, pseudoreversible photochemical reaction, Hecht (1934, 1935) has shown that for the cones, intensity discrimination may be described by the equation

$$\Delta I/I = C(1 + 1/[KI]^{\frac{1}{2}})^2 \tag{1}$$

When plotted on logarithmic coordinates this equation gives the curves used in this paper   Only the position of the curves on the ordinates will be affected by changes in the constants K and C Hence, the values of some of these constants must be different under varying experimental conditions, and the effects of area, surround, wavelength, fixation, and criterion can probably be understood in terms of their effects upon these constants   This is the point of departure for further analysis of such effects

It is necessary to add that only the cone data in our measurements are adequate for judging the validity of equation (1)   The rod data cover too small a range for a critical decision between it and the simpler form

$$\Delta I/I = C(1 + 1/KI) \tag{2}$$

The curves here drawn for the rods are the same as for the cones

Equation (1) is noteworthy not only because it describes the data for the human eye, but also because together with the related equation (2) it may be used equally well to express intensity discrimination for the bee, for *Drosophila*, and for *Mya* (Hecht, 1935)

The fundamental assumption that a difference in rates rather than in equilibrium quantities is the determining factor in intensity discrimination is reasonable if the photoreceptor system is a cyclical process, rather than self-contained and completely reversible   In such a process at least one of the photolytic products will be continually dissipated, either in the secondary reaction which follows excitation, or by diffusion away from the place where it is effective   The effective rate at which such a material is supplied is *the rate of the photochemical part of the reaction system*, and the kinetics of the dark process and the stationary state condition merely control the concentration of the sensitive substance which absorbs the light

The least satisfactory part of the derivation is the dependence on *initial* difference in rates rather than the final adapted difference between two rate levels   When the data are obtained by the method of moving stripes, as they are with insects, the assumption that these initial rates are important is realistic enough   However, data with the human eye, obtained with persistent awareness of a difference in brightness, *does not easily seem to represent the result of momentary,*

impermanent changes Against this feeling one may advance the argument, as Hecht has done, that persistent ocular movements renew the sensation continually In the absence of an adequate surround, these same ocular movements prevent the attainment of complete retinal adaptation, as we have seen

These conclusions are strengthened by the fact that efforts to de scribe the data by equations in terms of a constant difference of adapted rather than instantaneous rate levels have been consistently unsuccessful Moreover, all attempts to derive a theoretical photo chemical basis for the data on a variety of different assumptions yielded distinctly unsatisfactory functions, and have emphasized the peculiar adequacy of equation (1) for describing the greater part of the measurements

<div align="center">VI</div>

<div align="center">SUMMARY</div>

New measurements of the brightness difference sensibility of the eye corroborate the data of previous workers which show that $\Delta I/I$ decreases as I increases Contrary to previous report, $\Delta I/I$ does not normally increase again at high intensities, but instead decreases steadily, approaching a finite limiting value, which depends on the area of the test field and on the brightness of the surrounding field

On a logarithmic plot, the data of $\Delta I/I$ against I for test fields below $2°$ are continuous, whereas those for test fields above $2°$ show a sharp discontinuity in the region of intensity in which $\Delta I/I$ decreases rapidly This discontinuity is shown to divide the data into predominantly rod function at low intensities, and predominantly cone function at high intensities Fields below $2°$ give higher values of $\Delta I/I$ at all intensities, when compared with larger fields Fields greater than one or two degrees differ from one another principally on the low in tensity side of the break Changes in area above this limit are there fore mainly effective by changing the number of rods concerned This is confirmed by experiments controlling the relative numbers of rods and cones with lights of different wavelength and with different retinal locations

At high intensities $\Delta I/I$ is extremely sensitive to changes in bright

ness of surrounding visual fields, except for large test-fields which effectually furnish their own surrounds   This sensitivity is especially marked for fields of less than half a degree in diameter   Although the effect is most conspicuous for high intensities, the surround brightness seems to affect the relation between variables as a whole, except in very small fields where absence of a surround of adequate brightness results in the distortion of the theoretical relation otherwise found

The theoretical relationship for intensity discrimination derived by Hecht is shown to fit practically all of the data   Changes in experimental variables such as retinal image area, wavelength, fixation, and criterion may be described as affecting the numerical quantities of this relationship

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ON CRITICAL FREQUENCY AND CRITICAL ILLUMINATION FOR RESPONSE TO FLICKERED LIGHT

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I

The examination of the data of intensity discrimination has demonstrated that a just detectable increase of illumination, $\Delta I = I_2 - I_1$ (I_1 fixed, I_2 adjusted), is directly proportional to its standard deviation in all homogeneous series of measurements for which data are available (Crozier, 1935–36)   This fact is of fundamental consequence for the interpretation of the meaning of measurements of intensity discrimination   It has been shown that with suitable experimental procedures the data of visual acuity and of flicker fusion may be regarded from the same standpoint, both are based upon phenomena of intensity discrimination and σ_I here has the properties of σ_{I_1} ($= \sigma_{\Delta I}$) (Crozier, 1935–36)   One consequence of these considerations leads to the prediction (Crozier, 1935–36) that a curve relating flicker frequency to mean critical illumination for threshold response to flicker will not be duplicated, with the same organisms, by the curve relating illumination to mean critical flicker frequency for the same response This results from the fact that the regression of mean flicker frequency (F) upon illumination (I), as obtained from measurements of F as a function of I, is not of the same character as that for mean I upon F Another way of stating this is, that the law according to which variation in flicker frequency depends upon I (or F) is not the same as the law according to which variation in I depends upon F (or I) Neither curve based upon averages gives an adequate formulation of the observed probability that a determination of F or of I at any point will possess the mean value recorded in the curve

The importance of this situation for the theoretical utilization of measurements of intensity discrimination requires a direct examina

tion of this particular case, which can be made more easily than that presented by direct tests of simple intensity discrimination, for purely technical reasons   The essential point concerned is that in the use to which the quantity ΔI must be put in deriving a physical theory of the excitatory process the actual dimensions of this quantity, as they arise in the experimental procedures employed, must not be lost sight of   A development of this matter will be found in a succeeding paper

It is to be borne in mind that the predicted lack of agreement between the "flicker curves" determined in the two ways indicated involves not only the mere fact of their different positions upon the F, I grid, the curve for mean values of F being expected to fall above that for mean I's (Crozier, 1935–36), but implies also certain specific quantitative features of relationships between the indices of dispersion of the measurements   These we may consider more advantageously after the technical procedures have been discussed

II

The reaction of a fish to a movement of a stripe system surrounding its container has been described previously (Lyon, 1904, Grundfest, 1931–32 a, b, Wolf and Zerrahn-Wolf, 1935–36) and has been used for the determination of sensitivity to visual stimulation

For observation a fish is placed in a cylindrical glass jar, 10 cm in diameter, containing 220 cc of water   The container with the fish stands on a glass-topped table   It is surrounded by a glass cylinder on which black opaque paper stripes are pasted, leaving translucent spaces of equal width between them   The striped screen is mounted on an axle which can be driven by a motor at various speeds (cf Fig 1, Wolf and Zerrahn-Wolf, 1935–36)

The striped screen is viewed by the fish against a white reflecting surface   This is a hollow 45° cone made of sheet metal and painted with zinc oxide   The cone is illuminated from below   The light comes from a source consisting of 100, 500, 1000, or 1500 watt concentrated filament lamps, according to the brightnesses desired, and placed at different distances on an optical bench   The positions of the sources are fixed distances from a diffusing screen (D) at the end of the optical bench   Behind the diffusing screen there is placed a diaphragm which controls the size of the radiating area of the screen   The light then falls on a mirror which is inclined at an angle of 45° and reflects upward through the glass top of the table to the cone   The intensities of illumination at the eye of the fish are measured by a Macbeth illuminometer   With the different light sources and various distances from the screen, and the diaphragm, the brightness can be varied over a range of 5

logarithmic units For low intensities we had to place in front of a 100 watt lamp in a fixed position Eastman Kodak neutral filters with transmissions of 1/10 1/100 1/1000 and 1/10 000 thus enabling us to have a total range of 9 logarithmic units of intensity

In a previous paper (Wolf and Zerrahn Wolf 1935–36) the reaction of the sun fish *Lepomis* to flicker was studied by adjusting the speed of rotation of the striped screen so that certain constant flicker frequencies were obtained A given flicker frequency was kept constant and the intensity of illumination was changed by increasing I until a threshold reaction of the fish became evident For the present
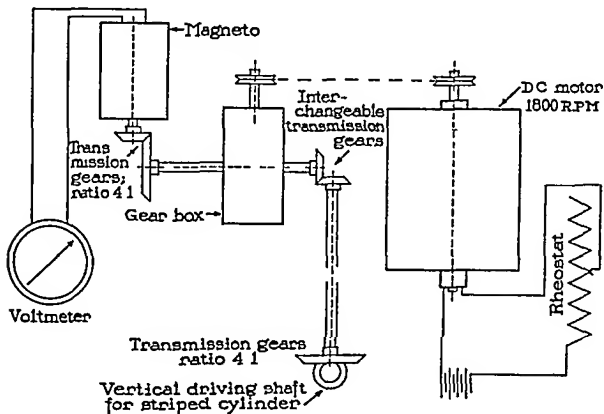
FIG 1 Diagram of connections of driving motor with shaft for rotation of striped cylinder and with magneto for measurement of speed

study it was desirable to take as the fixed intensities the mean values for threshold intensity of reaction from the previous experiments The flicker frequencies were varied by decreasing the flicker frequency until a threshold response of the fish is obtained The temperature throughout was 21 ± 1°

For determination of threshold flicker frequencies at fixed intensities it is essential that (1) the transmission of motion to the striped screen be rigid, so that no slippage of belts in the transmission system may influence the flicker frequencies in an uncontrollable manner and (2) instantaneous readings of flicker frequencies can be taken

The striped cylinder is driven by a D C motor (1800 R P M) the speed of which

is controlled by a rheostat (Fig 1)   The motor is belted to a reduction gear   The drive shaft of the gear box transmitting the reduced speed of rotation is on one side connected by a set of gears of ratio 1 1 to a shaft which transmits the motion through another set of gears of ratio 4 1 to the shaft turning the striped cylinder The gears at the gear box junction can be interchanged for another set of ratio 2 1, to produce higher flicker frequencies in case they are needed   The gear transmission has no free play, and accurate settings of speeds of rotation are thus possible
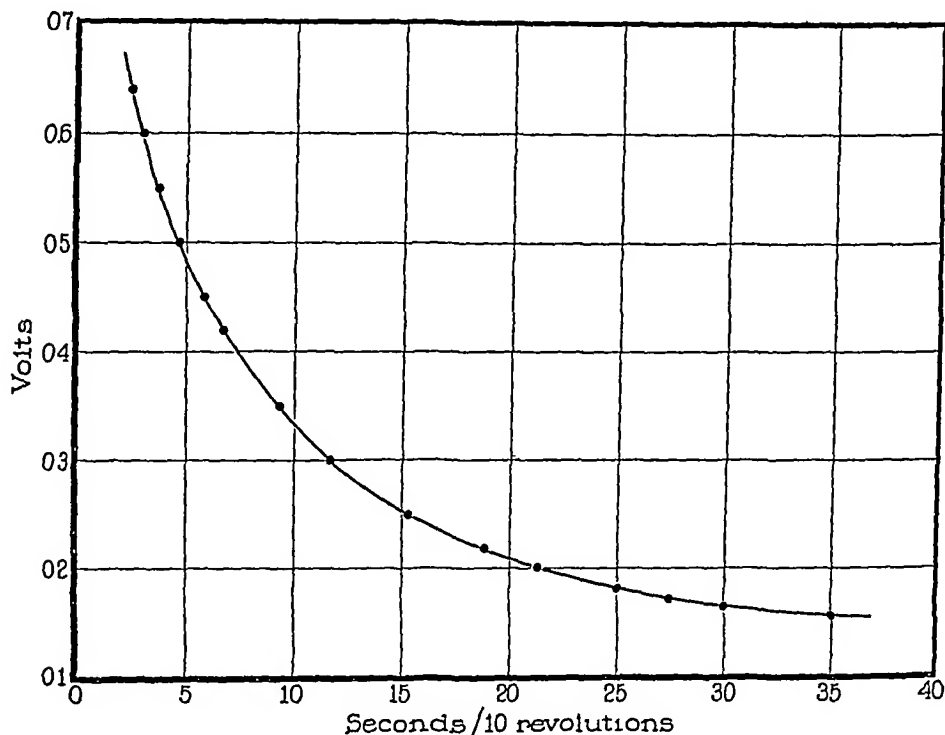
FIG 2   Typical calibration curve—voltmeter readings from magneto as related to speed of rotation of striped cylinder

The other end of the gear box shaft is connected with a second set of gears of ratio 4 1, transmitting the rotation to a magneto speed indicator system   The magneto is connected with a millivoltmeter from which by means of calibration curves the flicker frequencies can be read   In the tables the voltages have been rounded to the nearest 0 01 volt

For calibration of the speed indicator the rheostat is set at a given position, the time for ten revolutions of the striped cylinder is determined by means of a stopwatch, and the voltmeter reading taken   Such determinations are made for a great many rheostat settings covering the entire range of speeds   The voltmeter

readings then are plotted as ordinates against the times for ten revolutions as abscissae (Fig 2)   We thus obtain a smooth calibration curve from which the flicker frequencies can be computed   Since for the entire range of flicker frequencies required, different striped cylinders have to be used (10 20, and 40 stripes each) the total number of stripes passing in front of the fish s eye during ten revolutions must be divided by the values from the calibration curve read on the abscissa for any given mean voltmeter reading to give the flicker frequency   For each set of gear ratios used a separate calibration curve is of course required   Only one of these is illustrated (Fig 2)   The different gear ratios are so chosen as to permit readings from the calibration curves in the region of maximum precision, rather than at either end of the curve (Fig 2)   In this way an approximately equal accuracy of instrumental estimate of flicker frequency is obtained over the whole range of frequencies used

For experimentation twelve individuals which gave rather good reactions to moving stripes were selected   They were of the same batch from which the animals for the previous experiments (Wolf and Zerrahn Wolf 1935 36) were taken   Each animal is kept in a separate glass jar   For tests they are transferred into culture dishes 10 cm in diameter and 5 cm high   Each dish is filled with 220 cc. of clean tap water which was at room temperature before the fishes were put into it.

Before experimentation the fish are dark adapted for at least 2 hours   The first animal is then placed on the glass top of the table and left in the dark for a short time, so as to avoid any interference with our first reading by any effect of the handling   Then the striped screen is set into motion at a speed which is considerably higher than that at which the fish could be stimulated by the flicker produced   The light is turned on giving an illumination previously adjusted to the desired intensity   As soon as the light is turned on the fish shows a slight light 'shock' which consists in a sudden backward motion after a few seconds it becomes quiet again   Then the position of the rheostat is changed (decreasing the flicker frequency) until the fish shows the first noticeable response   At this instant the voltmeter reading is taken by another observer   Since the fish now is swimming forward with the stripes or backward against them a shutter is closed reducing the light to a minimum   Then the speed is brought back to the original maximum level and as soon as the fish has become quiet again a second and a third test is made   With all animals and at all levels of intensity we took three successive readings which agreed very closely one with another

Since the transmission of rotation to the striped screen was done by a rigid gear system there is naturally some vibration of the jar in which the fish is kept   The fish therefore is never so quiet as when the transmission is by a belt system   The fish always shows active motions of the fins   It stays reasonably quiet however, and the first jerk it gives with the visual stimulus produced by flicker is sharp enough to give repeatedly accurate settings for critical flicker frequencies   The various intensity settings were made in random order

III

The analysis of the data obtained by measurements of mean critical illumination intensities (I_m) at fixed flicker frequencies showed that the dispersion of intensities (I_i) was related in a simple way to I_m, σ_{I_i} is directly proportional to I_m in the lower (rod) region of the curve, and directly proportional to $(I_m\text{-}const)$ in the upper (cone) section of the curve (Crozier, 1935–36)   This result was brought into harmony with the results in tests of intensity discrimination, where in general σ_{I_2} $(=\sigma_{\Delta I})$ is directly proportional to I_2, on the basis that reaction to flicker involves an "intensity discrimination" between I_x and $I_x -$ ΔI_x, where $I_x - \Delta I_x$ (the brightness effect of the dark stripe) $\equiv I_1$, and $I_x \equiv I_2$ The data are properly described not by a curve but by a band, the width of which measures the probability that repetitive determinations of critical I will fall in a particular area, the margins of the band may be fixed by $I_m \pm \sigma_{I_i}$, or by $I_m \pm \text{P E}_{I_i}$, or by σ_{I_m} or P E_{I_m}, since for each value of I_m the number of observations is kept constant   For the present purpose it is convenient to use $I_m \pm \text{P E}_{I_i}$ The data are summarized in Table III   The horizontal width of the band has the properties of $k\,\sigma_I$ Its *vertical* width should predict the properties of σ_F if the experiment is made by determining F as a function of I It is also apparent (Crozier, 1935–36) that the curve for mean F as a function of I should lie above that for mean I as a function of F (*cf* Fig 3)—unless the frequency distribution of measured F's or I's should be found to be of quite unexpected shape

This might easily happen if it were correct to regard the observed variation of I as in the crude sense due to "experimental error", error, that is, in the sense of variation introduced by the manipulation of the apparatus and of the organism with which the measurements are made   If this were correct, σ_I should be greater, absolutely, at low I's, and there would be no reason whatever for assuming that σ_I should be directly proportional to I, particularly since at high flicker frequencies (and high I) the reaction of the fish which is the basis of measurement is sharper, "cleaner', and easier to recognize quickly   The instrumental errors are thus in general to be expected to produce a type of dependence of σ_I upon I_m which is in fact the reverse of that actually encountered

It might also be suggested, in line with a certain more or less traditional conception of the "interval of uncertainty", that at any moment a given tested individual fish will exhibit a zone of intensities within which it cannot distinguish intensities at fixed F, and *vice versa*, and that in consequence any single determination of critical I or of critical F represents an accidental overshooting of the true margin of this zone   Several considerations dispose of this view   If this were in fact the essence of the situation then the distribution of measured I's at fixed F, or of measured F's at fixed I, should be quite definitely skewed, heaped up at the *low* intensity side and at the *high* F side, owing to the way in which the end point is approached   And it would be impossible to foretell from knowledge of the properties of σ_I anything whatever as to the properties of σ_F.

In opposition to this idea is the one which we employ, namely that a single determination of F_1 measures a state of the reacting system of the fish, which has a certain probability under the conditions imposed   The basis for this position requires brief consideration

Prediction of the *properties* of σ_F is not to be confused with prediction of its absolute magnitudes   The manipulative process whereby critical I is measured is not the same as that by which critical F is ascertained   Each process must involve its own characteristic factor of procedural uncertainty   The method whereby I_1, F_1 and mean I, F are obtained reduces to a relative degree of insignificance the systematic distortions otherwise introducible from this source

It is to be noticed, in the first place, that critical I is determined by increasing I to the point where reaction is obtained, while critical F is gotten by reducing F until reaction is given   The important feature of this is that the same reaction serves as indicator in both sets of measurements, and the same end point is in each case approached in an equivalent manner   Using the terminology already employed, reaction is evidenced when I_x, the direct excitatory effect ("brilliance") of the illuminated stripe, reaches a critical value in relation to $I_x - \Delta I_x$ This effect is a function of I and of t, the duration of exposure to a stripe ($= k/F$)   In measuring critical I, t is constant, in measuring critical F, I is fixed   Since $I_x = \phi\ (I,\ t)$, the critical excitation can be approached by increasing I or by decreasing F It does not follow, however, that the mode of depend

ence of the critical effect upon F is independent of I, and indeed there is good reason to expect that change of F would be less significant at fixed high intensities than at low (so that the *precision* of mean F at the highest intensities would be comparatively less than otherwise expected)

In these experiments, as already outlined, three determinations of critical flicker frequency were made with each fish at each intensity The average of these was then taken, and the mean of the twelve

TABLE I

To illustrate nature of the data upon which mean critical flicker frequencies are calculated The illumination I is fixed, cylinder with 40 stripes, the variation in mean flicker frequency from fish to fish greatly exceeds the variation in repeated determinations (voltmeter readings) with any single fish

log I (millilamberts)	Fish No	Voltmeter reading				Flicker frequency
		•			*mean*	
1̄ 8118	1	0 39	0 37	0 37	0 377	30 19
	2	0 38	0 38	0 37	0 377	30 19
	3	0 35	0 38	0 36	0 363	29 20
	4	0 39	0 37	0 39	0 383	30 89
	5	0 38	0 39	0 39	0 387	31 13
	6	0 37	0 39	0 38	0 380	30 53
	7	0 39	0 39	0 40	0 393	31 62
	8	0 40	0 40	0 39	0 397	32 13
	9	0 40	0 41	0 41	0 407	32 92
	10	0 38	0 39	0 40	0 390	31 50
	11	0 39	0 40	0 39	0 393	31 62
	12	0 39	0 40	0 39	0 393	31 62

individual averages used as the mean critical F This procedure tends to eliminate the purely instrumental errors, and is manifestly a legitimate procedure since the scatter of the readings with any one fish is quite markedly less than the variation from fish to fish Neither the raw single determinations of F_1, nor the individual means, are skewed in distribution There is no basis for supposing that the readings represent "overshooting" of the margin of a zone of uncertainty One set of typical results is given in Table I An examination of the data on critical I's (*cf* Wolf and Zerrahn-Wolf, 1935–36)

shows an entirely similar situation   The necessary presumption is that the different individuals represent, at any one time, an essentially random distribution of the capacity to be excited by the flickered light, as evidenced by the respective critical flicker frequencies obtained for each   This is amply substantiated by an examination of the relative positions of the twelve in tests at different intensities and at different times   The mean rank position (1 to 12) in terms of increasing critical flicker frequency for response was determined from

TABLE II

Relative sensitivities of twelve *Lepomis* in terms of mean relative position in the series (1 to 12) of increasing critical flicker frequencies in twenty three sets of determinations at various times and at different intensities

Individual No.	Mean position
1	4 55
2	4 69
3	5 14
4	6 86
5	6 76
6	6 76
7	8 42
8	6 76
9	8 01
10	5 76
11	6 91
12	7 12
Mean	6 48 ± 0 846

twenty three sets of measurements such as the one shown in Table I   The mean of these means was 6 48 (completely random = 6 5), the P E of a mean rank position 0 846, the maximum departure from the mean being 1 96 (see Table II)   The difference between the extreme mean rank positions = 2 3 × its P E   This is the relationship to be expected from a fluctuating chance distribution of relative reactiveness   The time fluctuation of reactiveness in one fish is of a totally different order of magnitude from that concerned in the narrower range of variation of F apparent in successive determinations with one individual at one intensity   The situation here is like that encountered

in measurements of geotropic orientation of rats (*cf* Crozier and Pincus, 1931–32, Crozier, 1935) different individuals of one genetic composition represent at a given moment different states of performance of the same reacting mechanism, the fluctuation of reactivity is random in each of the several individuals, over a range of which the properties may be measured by the performance of the several representative states of reactivity in the sample taken at one time For our present purpose the importance of this lies in the demonstration that the variation dealt with in the measurements is not instrumental error but is a property of the organisms and of the system of processes determining the effects measured   The use of a homogeneous population of tested individuals for all determinations in a series is merely a short-cut to the data which would be obtained by more protracted investigation of a single individual, involving an equal number of determinations   The variation indices secured therefore measure an essential aspect of the performance of the system under scrutiny

It is of particular interest to examine experimentally the behavior of the index of dispersion of F_1 as it may be affected by purely instrumental errors   Four independent sets of determinations illustrate this   (1) The first determination of mean critical flicker frequency was made at an intensity of antilog $\bar{4}$ 9850, it gave mean $F = 8$ 33, but P E $_{F_1} = 0$ 336   Subsequent determinations, after skill had been attained in repeated measurements, gave $F_m = 8$ 38, with P E $_{F_1} = 0$ 188   As pointed out subsequently, the latter determination is concordant with the position of this intensity in the whole series   (2) In a similar way, at a later date, it became necessary to change the gear ratio in the transmission system driving the striped cylinder, this entailed a new kind of practice on the part of the observer in using the rheostat controlling the speed of the driving motor, mean F from this series was 46 55 $\pm$ 0 929, after adequate training in the handling of the apparatus, a redetermination (Table III) gave $F_m = 46$ 46 $\pm$ 0 436, there is no question that the latter determination is a real measure of the variation effect   (3 and 4) We have described how in order to obtain an adequate range of flicker frequencies it was necessary to use cylinders with different numbers of opaque stripes, in two cases we have determined that when the number of stripes is

just barely inadequate to provide a sufficiently high flicker frequency to cover the range desired in the test, the mean flicker frequency obtained is, as would be expected, a little too low—for example at in-
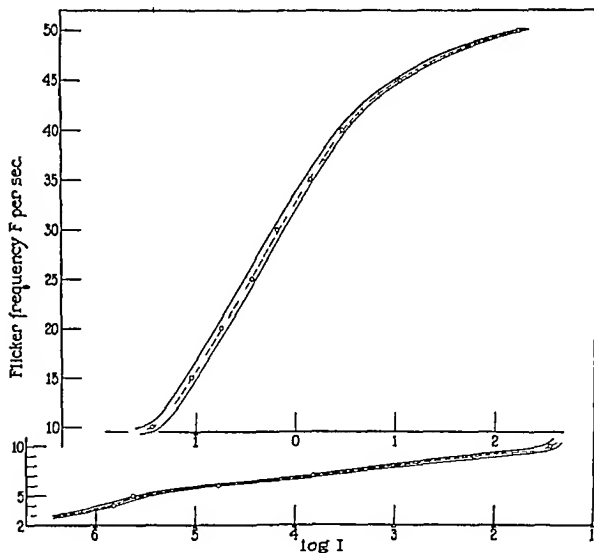
FIG 3 Critical I_m as a function of F (data from Wolf and Zerrahn Wolf, 1935–36) plotted as $I_m \pm P\ E_{I_1}$ to show form of the plot from which 2 P E $_{F_1}$ (the vertical width of the band) may be predicted as a function of I, see text   For convenience, the curve is shown in two sections which are continuous (cf Fig 5) each point is the average of three determinations upon each of the same twelve individuals   The mid vertical points on this band give the expected ' values of F_m as a function of I

tensity antilog $\bar{3}$ 7980, F_m was 9 14 with a 10 stripe cylinder, known to be just barely inadequate, instead of 9 32, and at intensity antilog $\bar{1}$ 2591, F_m was 20 51 with a 20 stripe cylinder, rather than 21 26 with an adequate (40 stripe) cylinder   Yet in each of these cases

P E $_{I_1}$ was definitely *low* when the cylinders contained too few stripes, —in the first case P E $_{F_1}$ = 0 244 rather than 0 253, and in the second case 0 210 rather than 0 388   The point is, that when the readings cannot be made by beginning with a flicker frequency definitely too high, and approaching the end-point by a sufficient decrease of flicker frequency, the only reactions which can be recorded as reactions to flicker are those of animals definitely in the less sensitive portion of their possible range, thus the effect of using a not sufficiently high
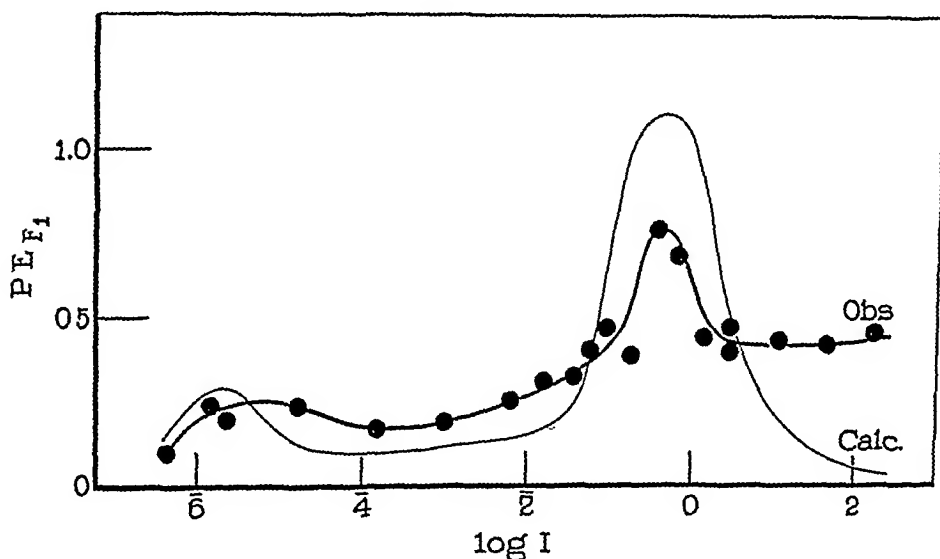
FIG 4   The half-width of the band shown in Fig 3, based upon P E $_{I_1}$, is taken as a measure of the expected dispersion (P E $_{F_1}$) of F The solid circlets are the experimentally determined values of P E $_{F_1}$, each point is the average of three determinations upon each of the same twelve individuals

range of possible flicker frequency is to produce an artificial kind of overshooting of the real critical frequency such as we have already commented upon in another connection, and it is significant that under such circumstances the index of dispersion of F_1 should be, as it is found, too small

On this basis we may expect to be able to predict certain of the properties of the observable variation of F In Fig 3 the band drawn embraces $I_m \pm$ P E $_{I_1}$ as a function of F The P E's have been adjusted to the smooth curve by means of the relationships discussed

in the preceding paper (Crozier, 1935–36)   One half the *vertical* width of this band, as a function of log I, is shown in Fig 4, it exhibits a minor and a major maximum   If the variation of I in determinations of critical illumination for response to flicker as a function of frequency reflects an organic property of the system excited, then the curve in Fig 4 should be in all essentials duplicated by the direct determinations of the variation of Γ in measurements of critical flicker frequency as a function of intensity   In the analysis of the data now secured it is manifest that the prediction has been successful

<div align="center">IV</div>

The results of the determination of F as a function of fixed I are given in Table III   The curve of F_m (Fig 5) is manifestly of the same general form as that secured by measuring I_m as a function of F, but it is definitely displaced upward, and the amount of the displacement is a function of I (or of F)   The *form* of the displacement can be predicted by computation from Fig 3   From Fig 5 the extent of the displacement can be gotten directly   (The original plots are of course very much larger)   The two are compared in Fig 6   Slight as the difference between the two curves may appear to be, it is thus none the less real and significant, since its somewhat peculiar major properties may be predicted   The separation of the two curves in Fig 5 is of the order of $-\mathrm{P\,E}_{I_1}$ at all intensities, but is about $-2\,5 \times \mathrm{P\,E}_{F_1}$, its consistency, however, removes this from the realm of accident   Moreover the form of the band (Fig 4) given by the dispersion of F_1 (measured by $\pm k\,\sigma_{F_1}$) is clearly like that already found from the measurements of σ_{I_1} (Fig 3)   This is to be examined carefully, since it involves the prediction of the form of σ_{F_1} from the data of σ_{I_1} and reciprocally   From Fig 3 the vertical width of the band may be computed, this should have the meaning of $2\,\sigma_{F_1}$ In Fig 4 it is compared with σ_{F_1} as found experimentally   (It is obvious that the converse calculation must also give agreement with the form of the measurements of $\mathrm{P\,E}_I$)

The chief source of disagreement between the curves drawn in Fig 4 may quite reasonably be found in the general fact that the F_m curve was determined some time later than the I_m curve   It has been pointed out, however, that an additional feature was recognized in

the fact that the method of securing a variable flicker frequency introduced the possibility of arousing the fishes through slight vibration, this would be expected to make F_m a little higher than in the absence of vibration It can be pointed out, however, that at the high intensity end of the curve P E $_F$ may be higher than predicted

TABLE III

Mean critical flicker frequency per second (F_m) and the P E of a determination of F ($= P E_{F_1}$) as a function of intensity (I), for the sunfish *Lepomis* These determinations are compared with the data for the same organisms in which mean critical illumination (I_m) has been determined as a function of flicker frequency (F) (*cf* Wolf and Zerrahn-Wolf, 1935–36, Crozier, 1935–36)   Each mean F and mean I is the average of three determinations upon each of twelve individuals

log I millilamberts	F_m	P E $_{F_1}$ ±	F	log I_m millilamberts	P E $_{I_1}$ ±
	per sec		*per sec*		
$\bar{7}$ 6550	3 70	0 0981	3	$\bar{7}$ 6555	0 694 × 10^{-7}
$\bar{6}$ 1750	4 09	0 240	4	$\bar{6}$ 1784	0 520 × 10^{-6}
$\bar{6}$ 3701	5 22	0 196	5	$\bar{6}$ 3701	0 399 × 10^{-6}
$\bar{5}$ 2300	6 42	0 239	6	$\bar{5}$ 2385	0 303 × 10^{-5}
$\bar{4}$ 1800	7 41	0 165	7	$\bar{4}$ 1855	0 279 × 10^{-4}
$\bar{4}$ 9900	8 38	0 188	8	$\bar{4}$ 9954	0 190 × 10^{-3}
$\bar{3}$ 7980	9 32	0 253	9	$\bar{3}$ 7983	0 124 × 10^{-2}
$\bar{2}$ 2000	9 79	0 311			
$\bar{2}$ 6600	10 69	0 327	10	$\bar{2}$ 5600	0 743 × 10^{-2}
$\bar{2}$ 7600	13 02	0 408			
$\bar{2}$ 9550	16 12	0 471	15	$\bar{2}$ 9543	0 126 × 10^{-1}
$\bar{1}$ 2591	21 26	0 388	20	$\bar{1}$ 2591	0 177 × 10^{-1}
$\bar{1}$ 5630	26 68	0 764	25	$\bar{1}$ 5631	0 630 × 10^{-1}
$\bar{1}$ 8118	31 13	0 683	30	$\bar{1}$ 8118	0 731 × 10^{-1}
0 1418	36 54	0 445	35	0 1418	0 129
0 4601	41 12	0 404	40	0 4601	0 281
	41 17	0 475			
1 0465	46 46	0 436	45	1 0465	0 415
1 6454	49 16	0 425			
2 2265	50 68	0 460	50	2 2264	11 53

because with high intensity and high flicker frequency a small change in F would be relatively less significant for excitation   One method of showing this is to consider the empirical equation which in general describes the course of such data, Hecht (1934) pointed out that the equation which may be written

$$kI = F^2/(C - F)^2$$
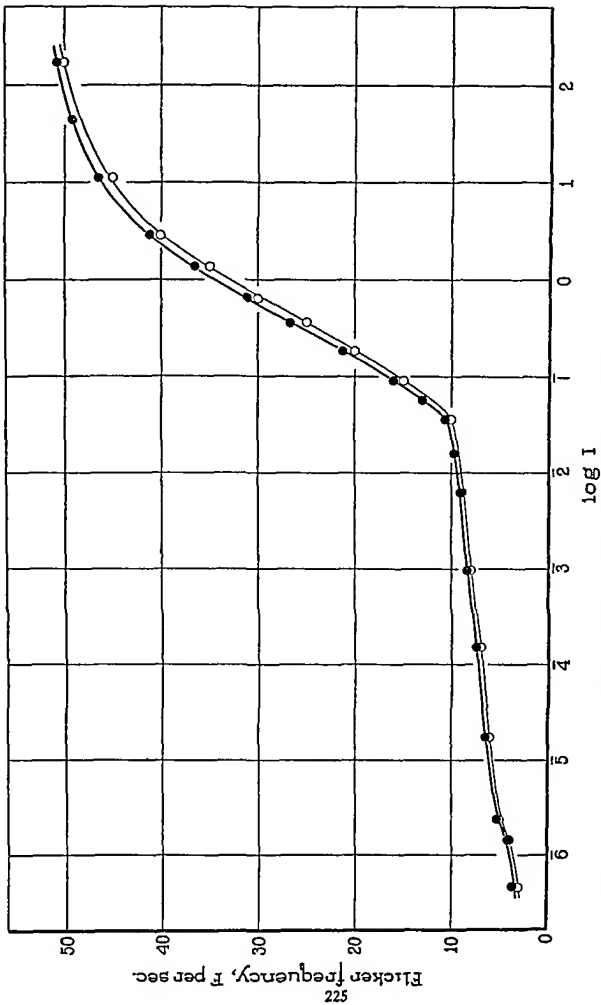
Fig 5 The curve of mean critical flicker frequency for response of *Lepomis*, upper curve, compared with the (lower) curve of mean critical illumination

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describes well the relation between intensity and flicker frequency for flicker *fusion* (cones), when I is large, a given change in apparent K calls for a relatively larger change in F than in I, this means that measurements of F_m at the highest intensities would be relatively less precise   It is a curious fact, however, that within the limits of the curves, the areas under the two graphs in Fig 4 agree to within 5 per cent   The only real agreement to be looked for, with regard to $P E_F$ observed and expected, is in the form of this quantity as a
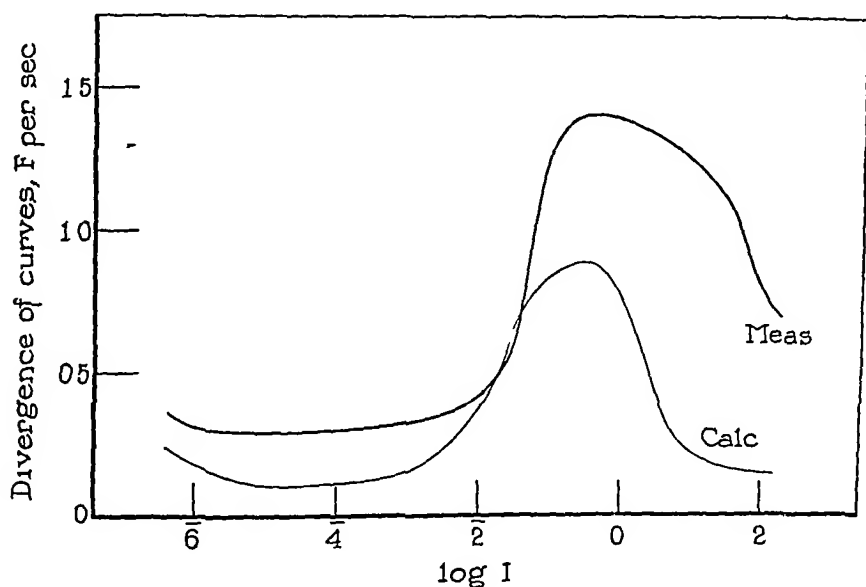
FIG 6 Expected (*calc*) and measured (*meas*) divergences of the two curves shown in Fig 5

function of I (or of F), the different procedures necessarily involved upon the one hand in obtaining F_m and on the other in getting I_m must be expected to result in minor differences in absolute amounts of variation   It is a reasonable surmise that this may be the origin of the effect noticed in certain other series of measurements, in which changes in the dispersions of the data appear to be correlated with the use of different procedures in attaining the end-point (*eg*, *cf* Hecht and Verrijp, 1933–34, Fig 1, and description in   Hecht, Shlaer, and Verrijp, 1933–34)

V

These considerations impose important restrictions upon the process of interpreting the theoretical meaning of a curve of thresholds for response to flickered light   In the first place, it is not correct to say that the *same* curve describes with sufficient precision the two sets of data obtained respectively by measuring mean flicker frequencies and by determining mean intensities   The quantities which may be calculated from such data and which may be used to test a theory of the visual excitatory process possess, and must retain, dimensions which describe properties of the reactions of the organism which are the basis of the measurements   The sensory phenomena, in terms of the index responses used for their estimation, are properly to be described by a formulation which embraces simultaneously the properties of σ_{F_i} and of σ_{I_i}, the dispersions of the measurements are properties of the reacting organism and not of experimental error   A quite general method of expressing this is to state that the law connecting flicker frequency and intensity, for threshold response, as found in the data, is to be represented as a band, *not* as a line or curve   The form of this band exhibits an essential property of the event whereby intensity discrimination is achieved   The use of this type of formulation in dealing with the prediction and the interpretation of other phenomena of photic excitation, and of comparable features of other kinds of excitation, will be considered in a following paper   A special feature of the data for response to flicker in the case of such a vertebrate as *Lepomis* is found in the superposition of effects due to excitation of cones upon those due to excitation of rods   The separation of these two effects presents certain complications   The whole situation may with profit be re-examined in the case of an organism in which these complications do not arise   A subsequent paper deals with the treatment of an analogous set of measurements made with such an organism

VI

SUMMARY

The curve of mean critical flicker frequency as a function of illumination has been determined for the reaction of the sunfish *Lepomis* to flicker   It exhibits expected quantitative disagreements with the

curve of mean critical illumination as a function of flicker frequency in the same organism   The form of the dependence of the variation of critical frequency of flicker upon illumination can be predicted from a knowledge of the way in which variation of critical illumination depends upon flicker frequency   It is pointed out that these findings have an important bearing upon the interpretation of the data of intensity discrimination

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THE EFFECTS OF CURRENT FLOW ON BIOELECTRIC POTENTIAL

III Nitella

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The effects of the flow of direct current on the bioelectric potential of *Nitella* include (a) polarization, (b) stimulation, (c) recovery or restoration   Some of these have been described, directly or implicitly, in a previous paper dealing with the direct current resistance of *Nitella* cells [1]

It was there noted that the high apparent D C resistance of the protoplasm is accompanied by, and is probably largely due to, a counter E M F built up by the current flow, which means that the previously existing bioelectric potential has been altered to that extent Such regular counter E M F 's will be designated "polarizations" for convenience, without prejudice as to their real nature (*i e* whether their time course corresponds to a static capacity or a polarization capacity, although evidence points to the latter)

The profound changes of P.D occurring during stimulation were also briefly described,[1] and have been the subject of many papers by Osterhout and co workers [2]   The present emphasis will be upon the current flow characteristics (threshold density, duration, direction, etc) which initiate stimulation, and the changes of polarizability which occur during its course

Finally, when the bioelectric potential has been greatly lowered, and polarizability lost, either briefly during stimulation or more permanently by treatment with KCl, current flows of proper direction and density restore the normal P D and polarizability   These may be called restorative effects

[1] Blinks L R., *J Gen Physiol* 1929–30 **13**, 495
Osterhout W J V *Ergebn Physiol* 1933 **35**, 967

These phenomena will be shown as changes of the normal bioelectric potential recorded continuously from string galvanometer deflections before, during, and after current flows of designated value, much as with *Valonia*[3] and *Halicystis*,[4] as published previously Many of the phenomena are common to all these organisms and will be compared as a basis for discussion of the mechanism of current flow effects

Methods

The electrical circuit was the modified Wheatstone bridge previously described [5] This balances the purely ohmic resistances of the system, while allowing potentials originating in the bridge (such as bioelectric potentials) to be recorded by the vacuum-tube detector feeding into the string galvanometer

Two differences of technique from the Valonia[3] *and Halicystis*[4] procedures may be noted   (1) Owing to the extremely high resistances of the delicate glass capillaries necessary for insertion in *Nitella* cells, and the difficulty of maintaining a supply of cells with these in place long enough for recovery to occur, impalement was not attempted   Two external contacts of fixed dimensions (1 cm long) were employed, consisting either of agar blocks imbibed with the desired solutions, as in the resistance measurements previously described,[1] or of solutions in the open ends of small glass U-tubes, slightly notched to hold the cells   When, as in most cases, it was desired to study the passage of current across only a single layer of protoplasm, one contact was treated with chloroform   This has been shown by Osterhout[2] to reduce the P D at that point approximately to zero, leaving that at the other end to record alone, and usually unaltered, for some time (15 minutes or more)   While a shunting path around this potential source is offered by the cellulose wall, the latter is very thin and is imbibed with tap water or distilled water so that its resistance between contacts is high—often 10 megohms or more [1] The discharge of the bioelectric potential (100 to 200 mv) through this resistance amounts to some 0 01 or 0 02 μa (2) Aside from this leakage, which is unavoidable with external contacts, there remains the possibility of discharge of the bioelectric potential through the bridge itself, which is of course a completed circuit This was found of negligible importance in *Valonia* and *Halicystis*, even through the low resistances ordinarily employed with these cells, but in *Nitella* the danger is more serious, since the discharge can itself easily become large enough to produce stimulation   Two methods are at hand to control it higher resistances in the bridge, or compensation of the potential   The former was simpler and was accomplished by using as ratio arms radio "gridleaks" of 1 or 10 megohms each   With the latter value the leakage through the bridge was reduced to the same magnitude

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[3] Blinks, L R , *J Gen Physiol* , 1935–36, **19**, 633

[4] Blinks, L R., *J Gen Physiol* , 1935–36, **19**, 867

[5] Blinks, L R., *J Gen Physiol* , 1929–30, **13**, 361

as that along the cell wall between contacts, making the total discharge, or residual current, not over 0 02 to 0 04 μa   Since the surface of the protoplasm at the standard contact of 1 cm length was of the order of 0 1 to 0 2 cm ^{2}, the residual current density was from 0 1 to 0 4 μa/cm ^{2} of cell surface, averaging about 0 2 μa/cm ^{2} This is usually insufficient to produce stimulation, although slight increments upon this value may be sufficient to do so, as will be seen

As a second method to reduce the residual current (that through the bridge) compensation of cellular E M F was produced with an equal and opposite E M F in series with the cell in its bridge arm   Sufficient records were taken in this way to show that there were no significant differences due to the extra current flow in the bridge over and above those through the wall   However, since such records no longer show the total P D, but only its changes they lose part of the advantage of working across a single layer of protoplasm   It is possible to correct this fault by introducing a second or pseudobioelectric" potential in series with the detector in the bridge diagonal   This "decompensates" the first compensating E M F, without introducing a new current flow because it is in series with an electrostatic instrument drawing no current   But such an arrangement is cumbersome necessitating frequent readjustment of both these E M F s to follow changing bioelectric potentials   If this adjustment is not made, current flow will be caused by the compensating E M.F at certain times e g as when the cell s P D has been reduced to a low or zero value during stimulation this might be more disturbing than the flow of residual current during normal conditions   All the records here published were therefore taken without compensation of the cell s potential just as with *Valonia* and *Halicystis*

Unless otherwise noted records usually refer to the same cell throughout each figure, and with minor excisions to conserve space, are continuous

The experimental current density values are marked in μa/cm ^{2} below each exposure on the records, an upward arrow signifying positive current passing inward across the protoplasm a downward arrow, positive current passing outward   These are of course the experimental increments or decrements effected on the residual current as a base   They are furthermore the increments or decrements which would have been produced if there were no counter E M F developed i e such as would have passed through the cell if dead or stimulated, or treated with KCl so that no counter E.M F appeared   They are therefore only transient initial values which are instantly decreased as the counter E M F develops, and their final value in the steady state may be considerably less e g a tenth or even a hundredth of the original value depending on the amount of counter E M.F developed   In some cases, the outside E M F was not applied directly to the bridge but through a high external resistance in series with it (e g 10 megohms)   This made charge and discharge conditions more nearly alike the charge occurring through this resistance the discharge through the ratio arms of equally high resistance   The results with this circuit were not appreciably different showing the time relations to be determined more by the cell itself than by the external resistances just as in *Valonia* [3]   This may be taken as evidence of polarization rather than static capacity

The bridge detector was the one-stage vacuum tube electrometer previously employed [5] In its plate circuit was balanced a Cambridge portable string galvanometer, which gives essentially faithful recording of potential changes, its response being nearly linear over the field employed, and practically instantaneous at the recording speeds used (about 1 cm per second)   The electrometer, working at the free-grid potential of the vacuum tube (earlier a 201-A type, more recently an 89 type, worked as a triode, for higher input resistance) drew practically no current and served as an electrostatic instrument responding to the potential drop along the part of the circuit which it tapped   In the bridge balanced with equal ratio arms its sensitivity was therefore reduced to one half its open circuit value, with the 10 1 ratio mostly used with *Nitella*, it had about 90 per cent of its open circuit value

Calibrations were introduced frequently on the records by an E M F in series either with the cell, or with the electrometer in the bridge diagonal   Calibrations in the latter caused no flow of current, but had to be corrected by the sensitivity factor (*i e* they were about 10 per cent higher than would be given by the same potential in the position of the cells)   Calibrations introduced in series with the cell caused of course a change of current through the bridge, hence altered the bioelectric potential, in the usual curved course   True series calibrations were obtained, however, at the end of each run when the cell was chloroformed, and from these are derived the millivolt ordinates inserted on each record   Here also the value of the basal, ohmic resistance could be checked, this was often about 50,000 ohms and was balanced by 500,000 ohms in the resistance arm   The absence of any deflection when currents were passed through dead cells (or a rectangular one if the balance was not quite exact), indicates that the charge and discharge curves obtained with living cells are really due to the protoplasm, and not to any spurious capacities in the electrodes or bridge circuit

The electrodes were non-polarizable, either Hg-calomel, or Ag-AgCl frequently recoated   Suitable salt bridges connected them to the cell contacts

The source of experimental currents was a tapped battery of dry cells, from which equal steps of voltage could be derived, the values applied to the bridge being determined by a potential divider and voltmeter, as previously shown [5]   With 10 cells in the battery, as much as 15 volts could be applied to the bridge, giving, through a dead cell of resistance 50,000 ohms, and a balancing resistance of 10 times this (due to the 10 1 ratio employed) a possible current of about 30 μa, or a maximum current density of 300 $\mu a/cm^2$ of cell surface   This is much larger than is ever necessary to bring about the effects here described, for which a maximum of about one-tenth this density is adequate in the most extreme cases (*e g* in the presence of KCl)   Usually not over 5 or 10 $\mu a/cm^2$ was passed, but this is some 10 to 50 times the normal residual current density

The temperature of the experiments ranged from 15 to 25°C , very few experiments being performed during the summer, when the cells are often difficult to stimulate [6]

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[6] Osterhout, W J V , *J Gen Physiol* , 1934–35, **18**, 987

A fall of effective resistance (disappearance of polarization) during the greatest depression of P D in stimulation was always found in the species of *Nitella* (mostly *N flexilis*) studied in New York with the technique described   This is in disagreement with the findings of Umrath who reports that there is no change of effective resistance during stimulation of the European species *N mucronata*   Umrath s results were obtained across a single layer of protoplasm by direct connection to the interior of the cells (impalement)   Current was pased from an inserted platinum electrode while a pair of non polarizable electrodes tapped the P D   Although polarization at the platinum electrode admittedly o scured the results Umrath s Figs 9 10 and 11 show a clear increase of its current during stimulation   This increase is greater for larger currents which would not be true if it were due solely to the P D change   Umrath s concurrent P D records are difficult to interpret since the purely ohmic IR drop of the system was not compensated

Aside from these technical points it is possible that the resistance of Umrath s impaled cells was already low so that the change during stimulation was not as great as in the present cases   It was previously emphasized[1] that *Nitella* cells must be very carefully handled, to display their highest resistances   Impalement may therefore introduce an injury which is only slowly recovered from   This is the case in both *Valonia*[2] and *Halicystis*[4] whose impaled cells for a long time display a much lower polarizability than intact cells   Recent measurements on the California species *Nitella clavata* have shown also that the effective resistance even of intact cells may sometimes be low so that little change of polarization occurs on stimulation although the P D still alters in characteristic fashion   Umrath s cells may have been like these   Polarizability and P D changes need not therefore always go together although they invariably did so in the records here presented and in hundreds of others like them   It is possible for example that polarization occurs largely at one surface of the protoplasm while the high P D originates at another   The two could be altered simultaneously or separately   somewhat as the sources of P D in *Halicystis* may be individually affected [8]

Another objection which might be fairly brought against the present technique is that electrolytes could diffuse out from the cell during stimulation and lower the resistance of the shunting cell wall   While this is entirely possible it would probably not occur as suddenly and *completely* as the observed changes and be as rapidly reversed again on recovery   In any case if the electrolytes came from the cell sap then the resistance of the protoplasm itself would probably be suddenly lowered to release them   The experiments of Sen [9] interpreted as showing such diffusion of electrolytes out of *Nitella* cells during repeated stimulation are ambiguous for the cells themselves were included in the measuring circuit and the observed resistance changes may have occurred in them   rather than in the adjacent

Umrath K *Protoplasma* 1932 **17**, 258

[8] Blinks L R Rhodes R D and McCallum G A *Proc Nat Acad Sc* 1935 **21**, 123
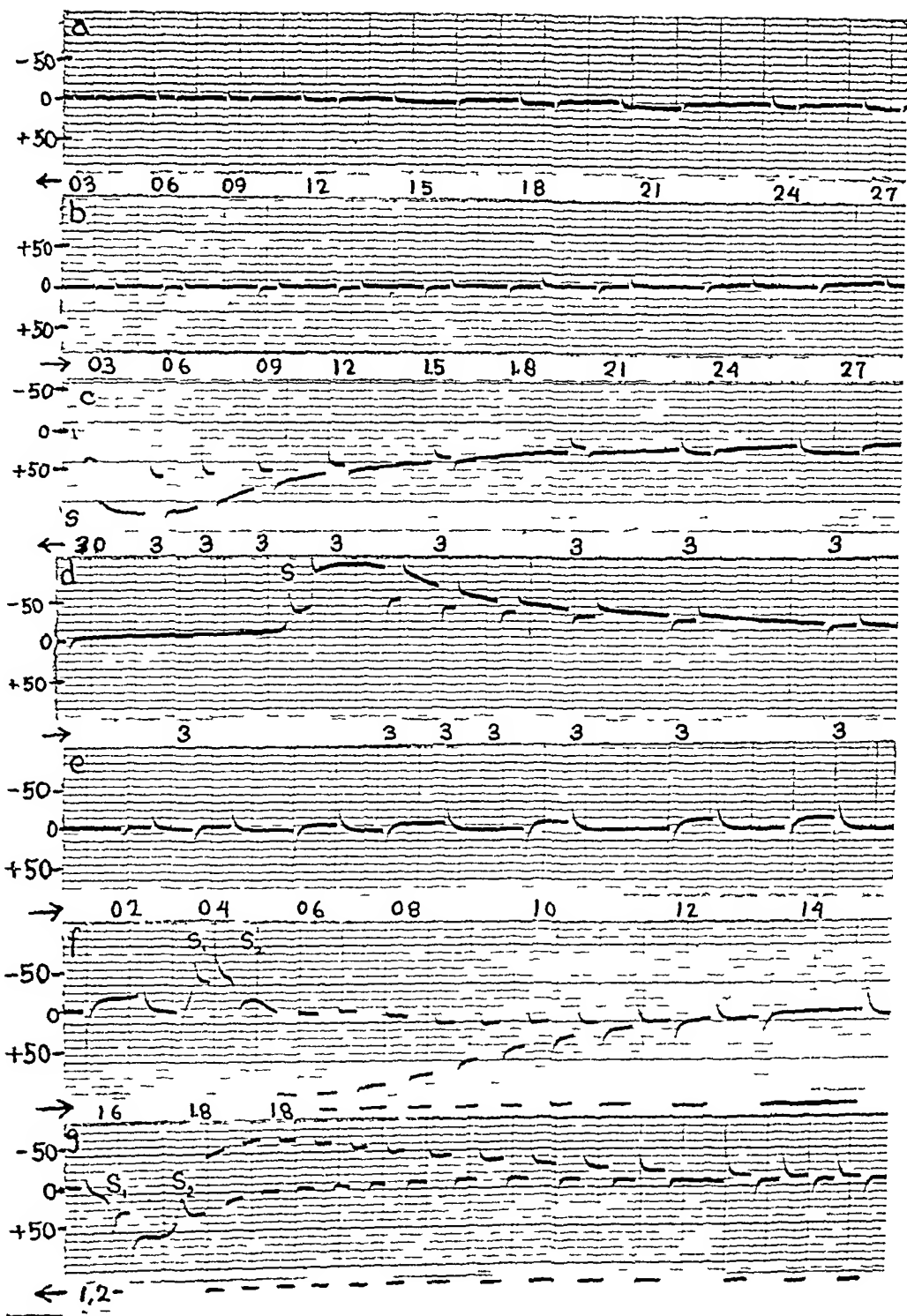
[9] Sen B *Ann Bot* 1931 **45**, 527

Fig 1

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Fig 1 String galvanometer records showing charge and discharge curves of counter E M F resulting from the flow of direct current through an intact cell of *Nitella* held between two aqueous contacts (tap water no chloroform) The bridge is balanced to the effective D C resistance of the cell here about 1 000 000 ohms (due mostly to the counter E M F) Current densities are designated in micro amperes per cm of cell surface (values at the beginning of current flow, before counter E M F develops) The duration of current flow is evident from the deflections at make and break since the curve records the P D of the right end of the cell in relation to the left end, upward movements represent increased relative negativity of the right end downward ones increased positivity The arrows indicate the direction of current flow through the cell, in Fig 1*a* the current flows from right to left and thus passes inward across the protoplasm at the right end outward across that at the left Increased positivity of the right end and decreased positivity of the left end result from this as the counter E M F develops

In Record *a* the first upward movement of the curve is due to the make of the current as the counter E M F builds up the curve comes back to the base line (of resistance balance) Since the current at the right end is inward the counter E M F is outward, making the P D more positive at the right end the current is outward and reduces its positive P D When the current is broken the curve jumps downward and then comes back to the base line (of no current flow) as the counter E M F fades away The curve at break is nearly a mirror image of that at make successive makes and breaks of current for larger currents merely increase the size of the deflections (there is an increasing unbalance of the bridge toward the end of Record *a*) makes and breaks of current from left to right in Record *b* give similar curves except for reversed directions the left end is here becoming more positive and the right end less positive as counter E M F develops Up to about 3 0 $\mu a/cm^2$ only such regular make and break curves appear (Records *a* and *b*) At this current density however stimulation (*s*) occurs on Record *c* at the left end of the cell (where current flow is outward) The positive P D of the protoplasm at that point largely disappears leaving that at the right end to record unopposed at break It amounts here to some 120 mv it disappears during the course of 15 seconds as the positive P D is regained at the other end Upon this altered P D as a new base current flows now produce different effects At make there is a large deflection away from the base and the counter E M F developed is insufficient to bring the circuit back to balance the effective resistance is lowered about 50 per cent As the P D recovers and the base approaches zero the counter E M F becomes larger and restores the bridge balance The counter E M F s in the early part of the curve develop entirely at the right unstimulated end of the cell as the left end recovers they develop also there

Record *d* shows stimulation at the opposite (right) end of the cell, due to passage of current from left to right hence outward at the right end The effects are the mirror image of those in *c* In both *c* and *d* some polarizability (about 50 per cent) remains because only one end is stimulated in each case without propagation down the cell In *e f* and *g* another more sensitive cell is recorded where stimu

film of water intended to receive the electrolytes   In addition Sen applied re-
peated and severe induction shocks, which, as Umrath[10] comments, may have
seriously injured or killed the cells, instead of merely stimulated them, without
concurrent P D measurements this cannot be ascertained

More direct evidence is also available that such a mechanism cannot account
quantitatively for the observed changes in the present technique   A cell was
immersed for several minutes in 0 1 M NaCl, which does not stimulate the cells,
like KCl yet has nearly the same conductivity as the vacuolar sap (about 10 per
cent less)   During this time the salt must have diffused thoroughly into the wall
The cell was then removed, drained and measured in the usual way   While its
effective resistance was lowered, the decrease was only some 10 or 20 per cent (in-
stead of 90 to 95 per cent occurring during stimulation[1]) showing that the cross-
section of the cell wall is too small to offer such a low shunting resistance, even
when fully imbibed with a salt solution nearly like that of the sap   Polarizations
remained prompt and large, disappearing as usual only on stimulation, and re-
appearing on recovery   Unless, therefore, the electrolytes released during stimu-
lation have a far higher specific conductivity than the cell sap itself, this explana-
tion must be ruled out

Legend for Fig 1—Continued —

lation (s_1) passes rapidly down the cell (s_2) and both ends lose their P D and polari-
zability to recover nearly in phase, so that the base line remains close to zero
Here, however nearly all counter E M F is absent for 2 or 3 seconds, at make of
current   the image deflects well away from zero, due to unbalance of the bridge, in
a nearly rectangular course, without curvature   then as recovery occurs, counter
E M F develops and causes the deflections to approach, and eventually reach, the
base line   In each make   the deflection is at first identical, thus in *f* the curve
shows that the deflection drops to the lower edge of the record each time the cur-
rent is made   but lingers there less and less time as recovery occurs, back E M F
developing and bringing the curve quite to the base line   This is difficult to see in
the latter part of the record because the curve rises so rapidly   This cell had a
lower threshold for stimulation in one direction than the other (as often occurs)
hence the first rectangular and later transient deflections away from base line in
Record *g* are about 2/3 as large as in *f*, corresponding to the current densities
employed (as marked)

On close inspection of the actual records, the momentary transient deflection
at make is seen to go to the same spot as in the rectangular deflections during
stimulation   A large current thus begins to flow at make but is quickly reduced
by the developing counter E M F

Sensitivity about 10 mv per horizontal division as marked   Time marks 1
second apart   Current densities as marked in $\mu a/cm^2$, refer to the large transient
values at the instant of make before decrease by counter E M F   Repeated flows
of the same current density are designated in *f* and *g* by bars of proper duration

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[10] Umrath K , *Protoplasma* 1932 **16**, 631

Experiments with Intact Cells

Very regular curves characterize the building up of counter E M F during current flow, and its disappearance on the break of current A few examples of these were included in a paper on the resistance of *Nitella* [1] Fig 1 gives a wider range of the same sort taken with both contacts uninjured (tap water, no chloroform), and with the bridge balanced to the steady state, or effective resistance, here about 1 meg ohm   The curves therefore deflect sharply away from zero at make of current and approach the zero line as the counter E M F builds up, to remain there as long as the current flows (steady state)   At break they deflect again to the same extent as at make, but in the opposite direction, approaching the zero line in a regular curve as the counter E M F disappears   Deflections resulting from 9 or 10 equal incre ments of current are shown, first in one direction through the cell, and then reversed   A marked regularity and similarity of charge and dis charge curves, during successive exposures, and with different current densities, may be noted

Finally, however, at sufficiently high current densities, a stimulation (S) occurs (at the contact where the current passes outward across the protoplasm)   Not only is the P D altered, giving the characteristic negative variation", but the effects of current flow also become quite different   With the bridge maintained at the original balance (1 megohm), the "charge curve no longer fully approaches the base line (now the altered P D of the action current) during current flow, but flattens out somewhat above or below it depending on the direction of current flow, and only tends to come back fully as the P D itself recovers   Still more strikingly, if the stimulation is not confined to its point of origin (cathode) but succeeds in passing down the cell to the other contact, there is for a moment no counter E M F whatever developed during current flow, as shown in Fig 1*f*   The deflection is abruptly rectangular, with no curve at make or break, showing that the resistance of the cell is purely ohmic, and much lower than the high resistance employed to balance the effective resistance of the un stimulated cell   Then as the cell recovers from the stimulus at both contacts the deflections become more curved, approach closer and closer to the (balanced) base line during current flow and eventually reach it again
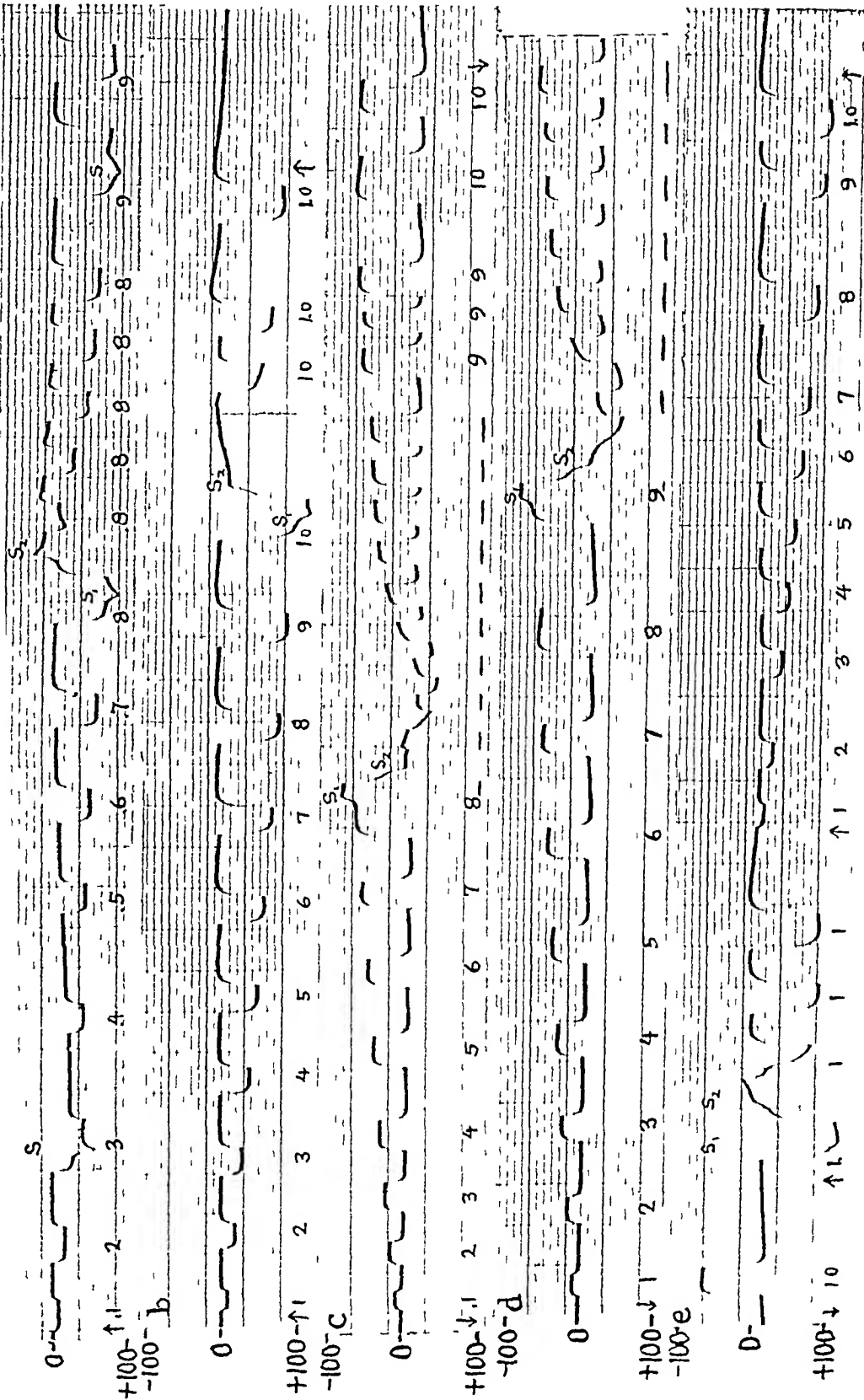
FIG 2

Records of somewhat different appearance but of essentially the same sort are obtained if the bridge is balanced, not to the effective resistance (which includes polarization) but to the true or ohmic resistance alone which is largely that of the vacuolar sap between the contacts Fig 2 shows records of this sort, still using an intact cell with both contacts normal Instead of starting as a sharp deflection away from zero, and then approaching it as the counter E M F builds up, the latter now starts at zero, and reaches a value above or below this base line corresponding to the direction and density of the current The curves, as regular and symmetrical as in Fig 1, are merely moved up or down in the field They show more clearly the proportionality of counter E M F to current density, up to the threshold for stimulation Here again the base line becomes distorted, due to loss of P D at one contact, and during this time the polarizations become of smaller magnitude if the stimulation spreads to the other contact they may become momentarily suppressed Since the bridge is balanced to the ohmic resistance of the cell, the loss of polarizability is shown by the decrease (or loss) of all deflection e g, Fig 2c Polarizability reappears as the cells recover from stimulation in the figure it appears to be slower than the recovery of P D, but this results from recording two intact regions, whose opposing potentials recover nearly in phase and so cancel each other Pictures truer in this regard, as well as in

FIG 2 Records showing charge and discharge curves of counter E M F developed by flow of direct current through an intact cell of *Nitella* much as in Fig 1 except that the bridge is now balanced to the true or ohmic resistance value (here about 50 000 ohms, largely due to the vacuolar sap) The counter E M F therefore builds up to its full value above or below the zero line depending on the direction of current flow and is nearly proportional to current density up to stimulation values when it is decreased, to build up again on recovery (here quicker than in Fig 1)

Current densities in $\mu a/cm$ upward arrows signifying passage of current from right to left downward arrows from left to right The potential is that of the right end which becomes positive to the left during the flow of inward current negative during outward flow and stimulates at a threshold of 0 8 to 1 0 $\mu a/cm$ (one stimulation occurs at 0 3$\mu a/cm^2$ in Record a) The bars in c and d signify the duration of repeated flows of current of the same density as the last previously marked

Sensitivity about 10 mv per horizontal division as indicated Time marks 1 second apart No residual current (except during stimulation) both ends being intact
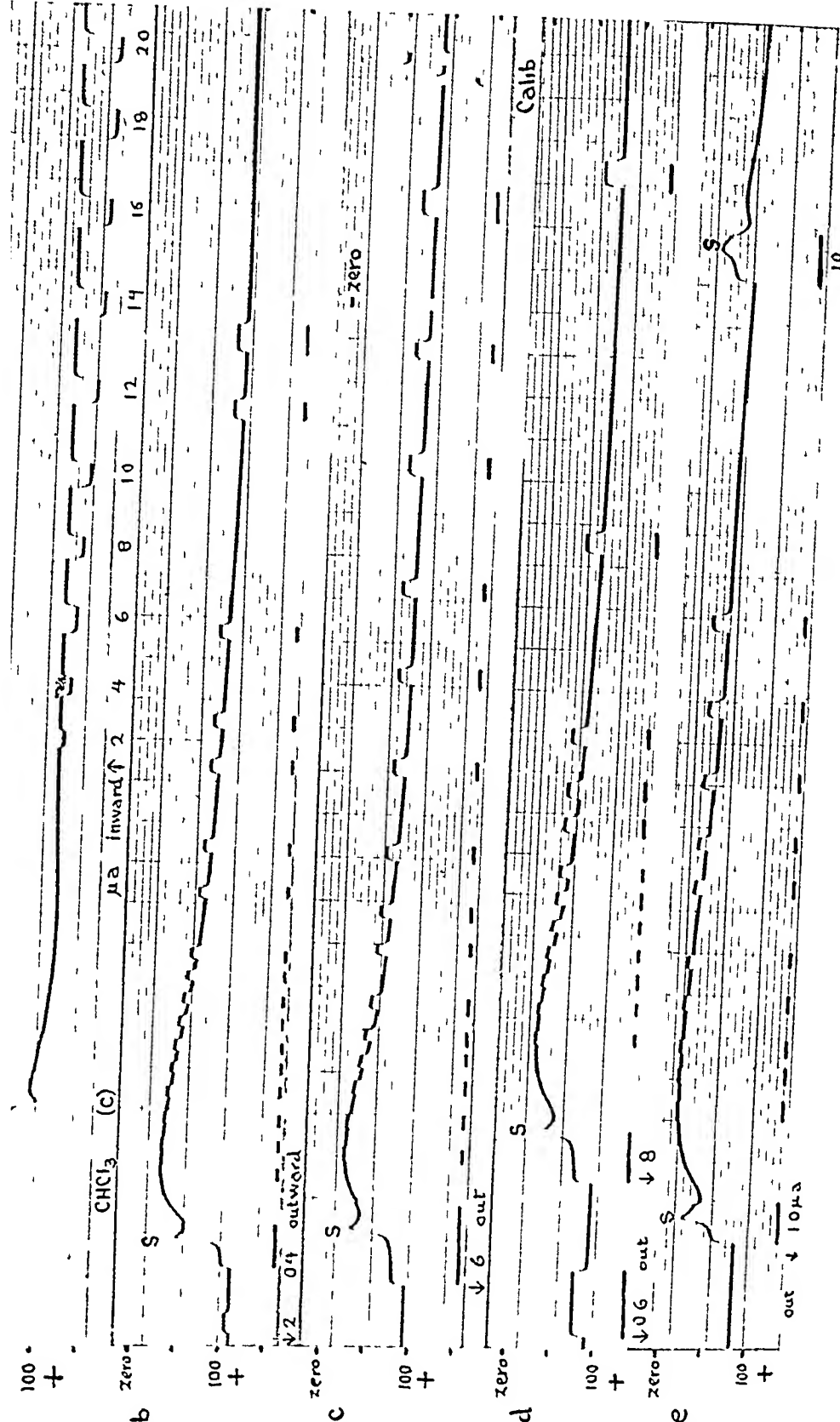
Fig 3

showing other directional effects without confusion, are obtained by recording only one protoplasmic P D , the other having been destroyed by chloroform

Experiments with One Contact Chloroformed

Fig 3 shows the characteristic change which occurs when chloro form has been applied to one end of the cell, owing to the disappear ance of potential at this point, the recorded P D rises from zero (now moved near the top of the record), to the full positive values of 100 to 200 mv which characterize the potential across the protoplasm of *Nitella* The cusp (C) is to be noted, it is almost invariably found both during the chloroform injury, and the action current or negative variation

Current is now passed, with the bridge balanced as before to the ohmic resistance of the cell   The same regular reproducible curves are found as before, but these now show definitely as increases or decreases of the original bioelectric potential, and it is possible to dis

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FIG 3   Records showing directional effects across one intact layer of protoplasm in *Nitella*   The zero has been moved to near the top of the records   Chloroform is then applied (CHCl₃ Record *a*) at the left end of the cell abolishing its potential and allowing that at the right to record in its entirety (about 120 mv)   The cusp (*c*) may be noted during this process it corresponds closely to that occurring during stimulation (*s*)   A current now passes continuously due to discharge of the bio electric potential through the bridge circuit this residual current is about 0 2 μa/cm   at the beginning and falls to about 0 13 μa/cm   in Record *e*

Record *a* shows the result of causing an inward current to enter at the intact region (right end)   counter E M F develops nearly proportionally to current density raising the total P D to 150 mv   Record *b* shows outward currents (superimposed upon the residual current)   these produce polarizations also but at a threshold of 0 4 μa/cm   (total 0 6 μa) a stimulation occurs the P D drops to about 30 mv , and polarization entirely disappears slowly reappearing   No further stimulation occurs at this density   but at 0 6 μa superimposed in Record *c* another stimulation occurs with the same characteristics   *d* and *e* show further increases of threshold with finally a second stimulation occurring at 1 0 μa in *e*

Current densities in μa/cm ² as marked bars indicating the duration of repeated current flows   Sensitivity about 8 mv   per horizontal division with calibrations as marked (a series calibration in *c* shows polarization charge and discharge due to current flow in the cell)   Time marks, 1 second apart
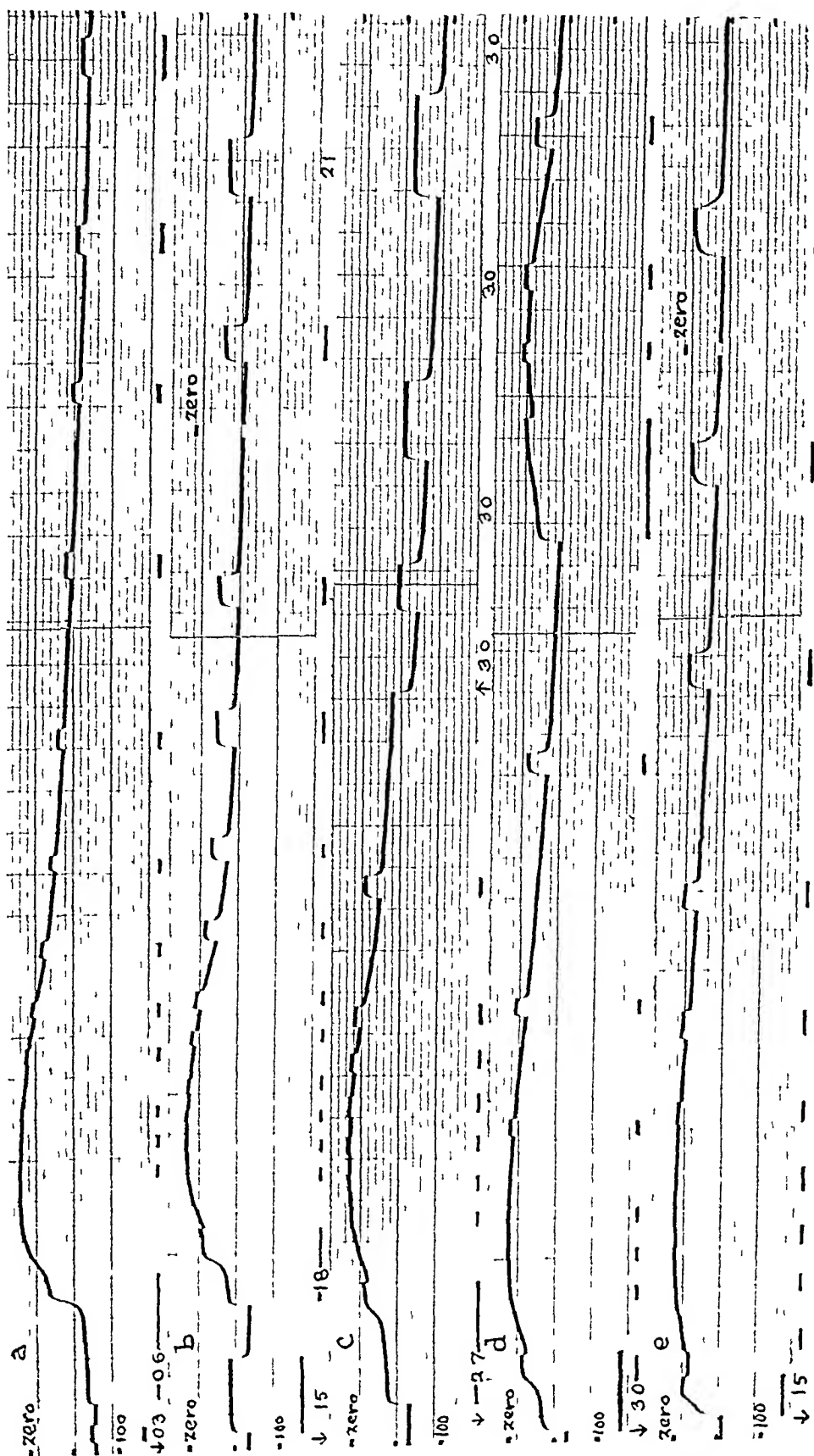
FIG 4

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tinguish between the effects of currents passing inward and outward across the protoplasm These may be taken up separately

Inward Currents —The counter E M F , opposing the flow of such current increases the existing potential, making it still more positive The amount of increase is nearly proportional to the current density up to fairly high values, sometimes to some 8 or 10 $\mu a/cm$ of cell surface although usually only up to 4 or 5 $\mu a/cm$ The P D may be increased thereby as much as 100 or 200 mv above its normal value, or to a total of some 300 or 400 mv   (This enhanced value is apparently the highest bioelectric potential yet measured in a single cell That in *Halicystis* seldom exceeds 100 mv , positive or negative,[4] and the small negative P D of *Valonia* may be reversed to 200, or at most 300 mv positive by the flow of current inward [2]) This appears to be an upper limit above which the P D cannot be further increased, even by much higher currents proportionality of deflections falls off above this, with the characteristic cusps and recessions shown in Figs 6 and 7 (Much the same situation holds for large inward current flows in *Valonia* [2])

Another effect of large current density, stimulation at the break of inward current flow, is best discussed after consideration of outward currents, since it is probably to be referred to the same mechanism

Outward Currents —These decrease the existing P D in regular curves comparable to the increases produced by inward currents   Examples are shown in Figs 3 and 4   The magnitude of change is again propor

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FIG 4 Records showing characteristics of stimulation and polarization much like those of Fig 3, except that the P D here goes to zero or slightly negative during stimulation   One end of the cell has been previously chloroformed incompletely at first, leaving a small P D which is responsible for the apparent negativity at the other end when stimulated   The threshold, at first 0 6 $\mu a/cm$ (and here showing a long delayed response) rises to 1 8, 2 7, and finally to 3 0$\mu a/cm$ (Record *d*) where a second stimulation occurs rather promptly   Finally after about 1 minute between Records *d* and *e* stimulation occurs again at 1 5 $\mu a/cm$ [2]

Note the disappearance of polarization during the maximum depression of P D and its reappearance during recovery

Currents are as marked in $\mu a/cm$ all outward across the intact protoplasm (downward arrows) except four flows of inward current in *c* (upward arrows)   Sensitivity about 8 mv per horizontal division with values as marked   Time marks 1 second apart   Duration of currents indicated by bars
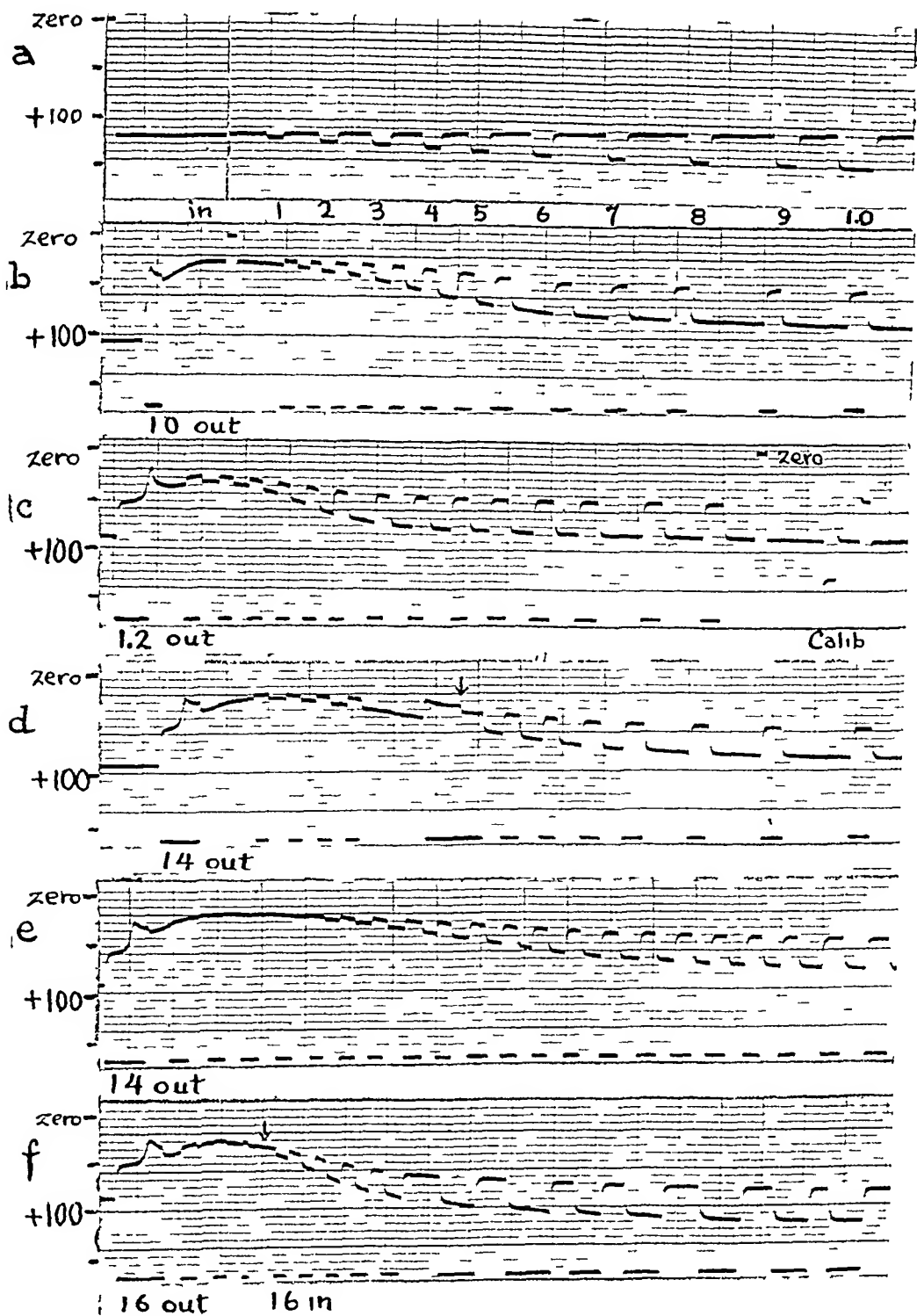
Fig 5

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tional to current density over a limited range, but the proportionality ceases at a much lower value than with inward currents, and with an opposite effect, since a much larger, instead of smaller, change of P D then occurs ("stimulation") The extent of proportional P D change varies from cell to cell, but is seldom over 50 or 60 mv (produced by a current of not over 0 5 or 1 $\mu a/cm$) With larger outward currents the curve inflects, instead of flattening out to a steady value, and the potential falls away to low values (as in Fig 3) or to zero

This curve is much like that produced by the flow of outward current upon the potential of *Halicystis*,[4] which also has a sigmoid course, and a cusp near the apex It differs, however, in the following respects

(*a*) The P D of *Nitella* seldom actually reverses in sign during stimulation, this may only mean, however, that in *Halicystis* there is a second (negative) source of potential (*e g* at the inner surface of the protoplasm) which is not affected by current flow, while in *Nitella* this either does not exist or is affected by current flow nearly simultaneously with the positive source

(*b*) In normal *Halicystis* a continued flow of current is necessary to carry the effects to completion, and to maintain reversed P D Stimulation in *Nitella*, once initiated beyond a certain point by the threshold current density, goes on to completion even if the current is stopped This is shown in Figs 3 4 and 5 where the current is interrupted at

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Fig 5 Records showing stimulation and recovery in *Nitella* with particular reference to the increase of threshold and the effect of inward current flow in speeding recovery The cell has been previously chloroformed at one end so that the P D is that across one contact This is proportionally increased in regular counter E.M F s to about 150 mv by the flow of positive current inward in Record *a* Prompt stimulation then occurs with 1 $\mu a/cm$ outward flow in *b* succeeding flows of this current produce at first no polarization (rectangular deflections being due to an ohmic unbalance (corrected at the arrow in *d*) then an increase The threshold rises to 1 2 $\mu a/cm$ in *c* 1 4 in *d* and *e* and 1 6 in *f* In the latter record the current is reversed from outward to inward at the arrow and with it the direction of counter E M F Note here that the recovery of positive P D is somewhat faster than in the preceding Record *e* where successive outward flows were passed

Sensitivity about 8 mv per horizontal division as marked and as shown by the 50 mv calibration in *c* (in series with the cell therefore producing current flow and polarization with a curved course) Time marks 1 second apart Bars indicate duration of current flows of the density previously marked

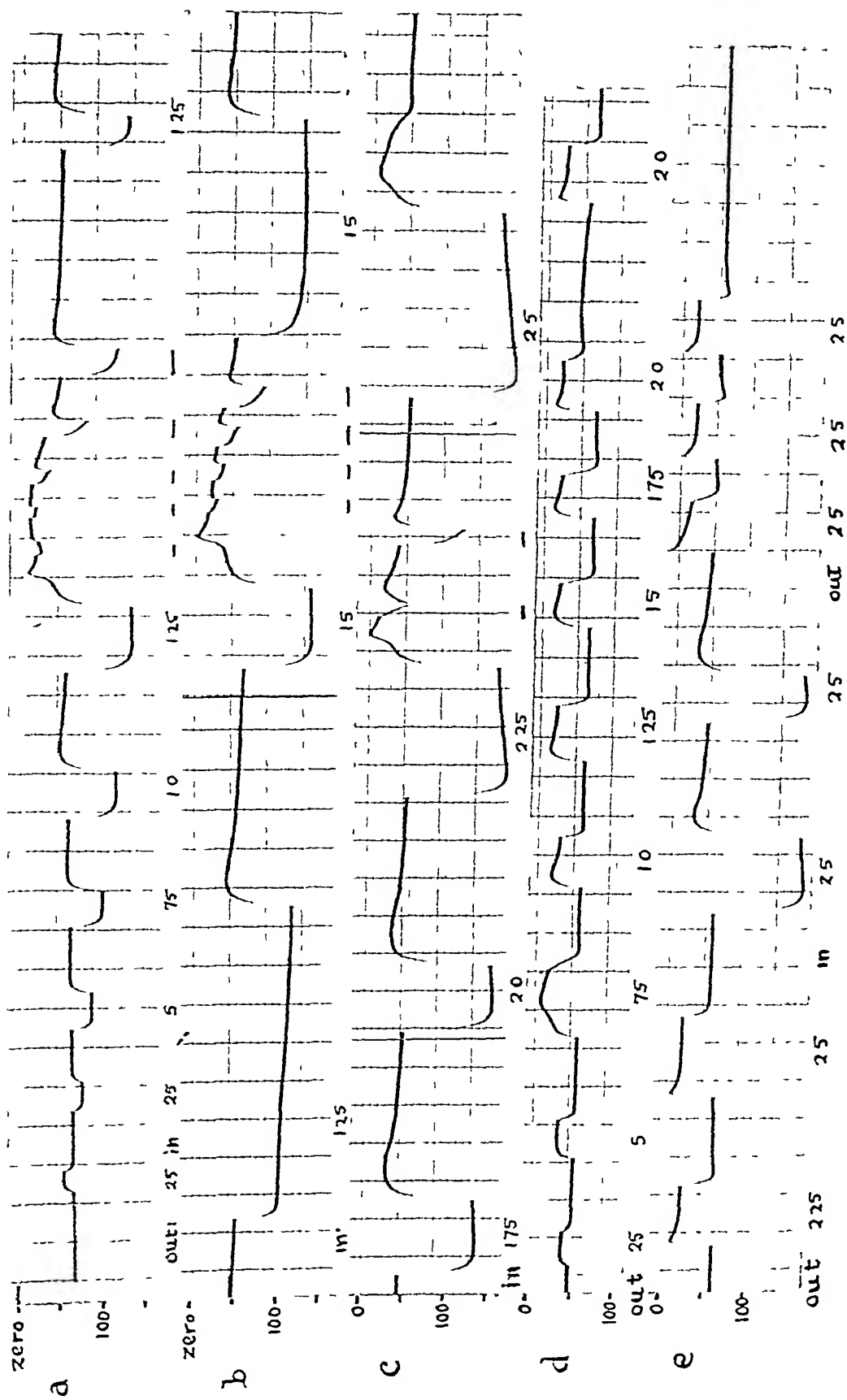Fig 6

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various points along the curve This is more like the situation in
Halicystis which has been treated with ammonia in concentrations
close to the critical reversal value [4]

(c) Recovery is also largely independent of the flow of current in
Nitella, often occurring even though the original stimulating current
continues to flow In *Halicystis* the P D remains reversed as long as
the exciting current flows (or even if it is considerably decreased in
value) positivity being regained only with much lower or zero outward
currents

The most striking change occurring during stimulation is the dis
appearance of counter E M F noted before with intact cells Figs 3,
4, and 5 show that as the P D reaches its lowest value, just after the
cusp, renewed passage of outward current produces little or no deflec
tion away from the base line, although the same current had just pre
viously produced a typical "charge' curve which preceded and passed
over into stimulation This absence of polarizability continues to
hold for a second or two, then as the P D begins to build up again,
charge and discharge curves also reappear, becoming larger as recovery

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FIG 6 Records showing polarization and stimulation in *Nitella* largely by in
ward currents with special reference to stimulation at break of the latter The
cell has been previously chloroformed in one contact so that the record is that of
the P D across the protoplasm at one contact only Small inward and outward
currents produce very regular polarizations in *a* At the break of 0 75 μa/cm
inward a very slight overshooting is evident this becomes more pronounced at
break of 1 0 μa/cm and passes over into characteristic stimulation at break of
1 25 μa/cm Succeeding currents of this density elicit at first small then increas
ing polarizations with finally an incomplete stimulation at the end of *a* (as shown
by the lessened polarization in *b*) In *b* at the increased threshold of 1 5 μa/cm
true stimulation again takes place on break of inward flow Increasing polariza
tions mark recovery but the threshold is now raised to 2 25 μa/cm in Record *c*,
where a break of 2 5 μa/cm finally causes a quick, rather incomplete stimulation

Outward currents are now passed again in *d* resulting in slight stimulation at
0 75 μa/cm [2] and again at 2 0 μa/cm but otherwise only in cusped polarizations
with overshooting on the break these occur both with inward and outward currents
in Record *e*

Sensitivity about 8 mv per horizontal division as indicated Time marks 1
second apart Bars indicate repeated flows of current of the density previously
marked
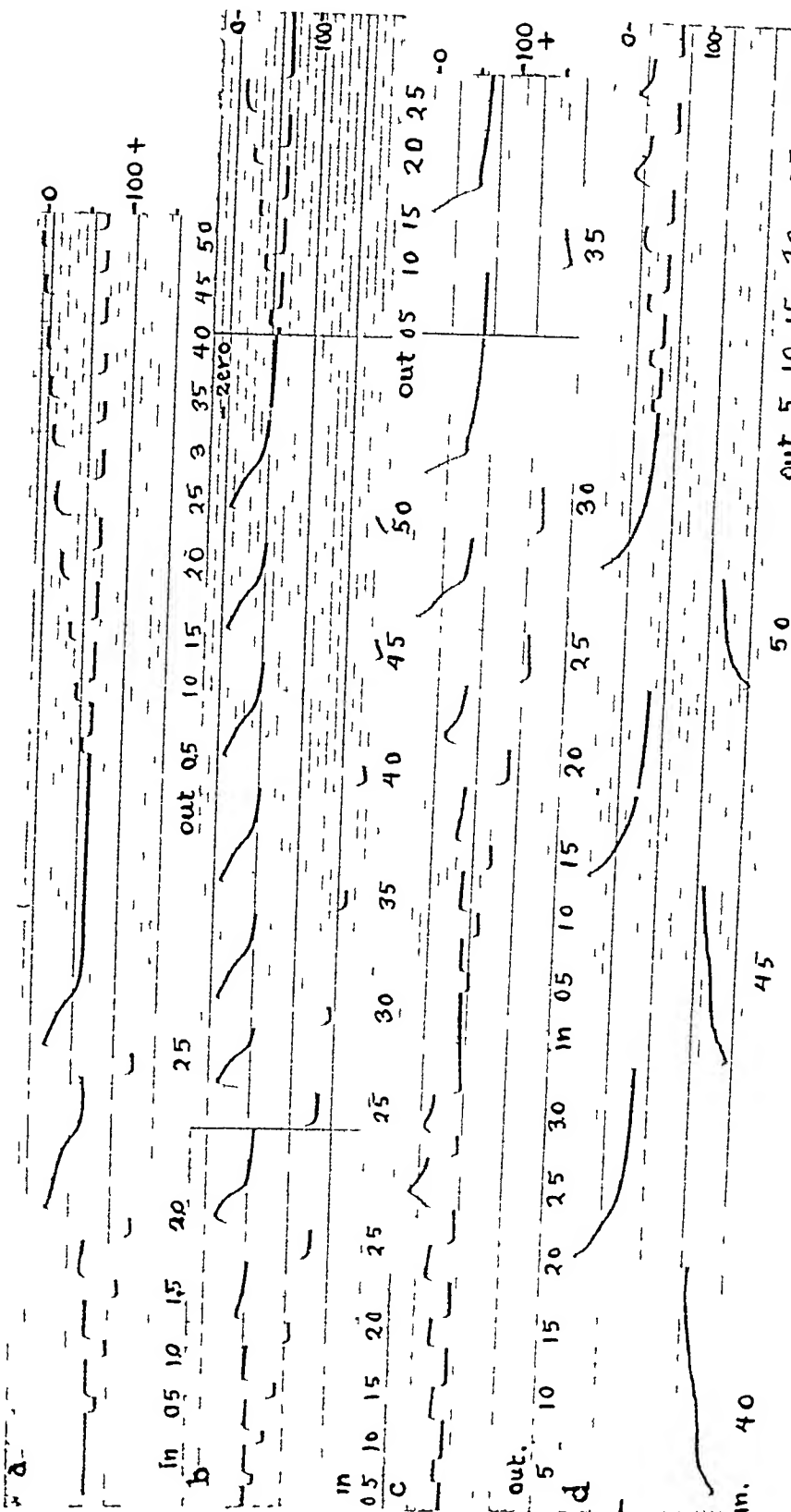
Fig 7 Records showing polarization and stimulation in a rather insensitive *Nitella*, particularly with reference to stimulation at break, and the sensitization to this produced by the previous flow of inward current Thus while stimulations are produced at break of 2 0 and of 2 5 μa/cm^2 inward flow, they do not occur over the range from 0 5 to 5 0 μa outward flow, where polarizations are regular and proportional up to rather high densities In Record *b*, break stimuli occur after every inward flow from 2 5 μa up, and again in *c* and *d*, while the stimulations resulting from outward flow are incomplete when occurring (e g, 2 5 μa/cm^2 in *c* and *d*) Cell previously chloroformed, so that p.p. is that somewhat

proceeds, and eventually reach their original size and shape   Polar
izability to inward currents is recovered *pari passu*, as shown in Fig 5*f*

Polarizability and a large bioelectric potential are thus closely
parallel in time, and may even have the same structural or metabolic
basis   This recalls the situation in *Valonia*, where very slight polar
izability is often displayed by cells in their normal low and slightly
negative P D level, while if this is made positive by acid sea water,
cresol, or the flow of inward current itself, more regular polarizations
also appear [3]

A further interesting behavior of *Nitella* is also shown in Figs 3, 4,
and 5 where a succession of identical currents were passed during and
after stimulation   It is seen that a second stimulation does not occur
with the same current density which produced the first, even some 30
seconds to a minute after recovery seems complete (*i e* , after the P D
has reached a constant high value)   Renewed stimulation occurs only
when a slightly larger outward current is passed, and this process may
be repeated several times with increasing current density   This
appears to be a real increase of the threshold for stimulation, for
although the P D itself may recover to a slightly lower positive value
each time, thereby decreasing the residual outward current which is
additive to the imposed currents in producing stimulation, this reduc
tion of residual current is not usually as great as the rise of additional
current necessary   It appears instead that the P D must be driven to
successively lower levels (*i e* , larger polarizations produced) by out
ward currents before stimulation occurs   Only at rather high current
density (1 0 $\mu a/cm^2$ in Fig 3, 3 0 $\mu a/cm$ in Fig 4), does a second
stimulation occur within 20 or 30 seconds

It is conceivable that the increased threshold for stimulation after
recovery is due to the same compensatory mechanism which causes
recovery itself (even during continued outward current flow)   There
might be produced a substance (*e g* an acid) which only slowly dif
fuses away or reacts, hence accumulates and requires a progressively
larger current to counteract its effects   This period of enhanced
threshold may be called a relative refractory period   There is also an
absolute refractory period, covering the greatest depression of P D ,
but also extending into part of the recovery phase, when even ex
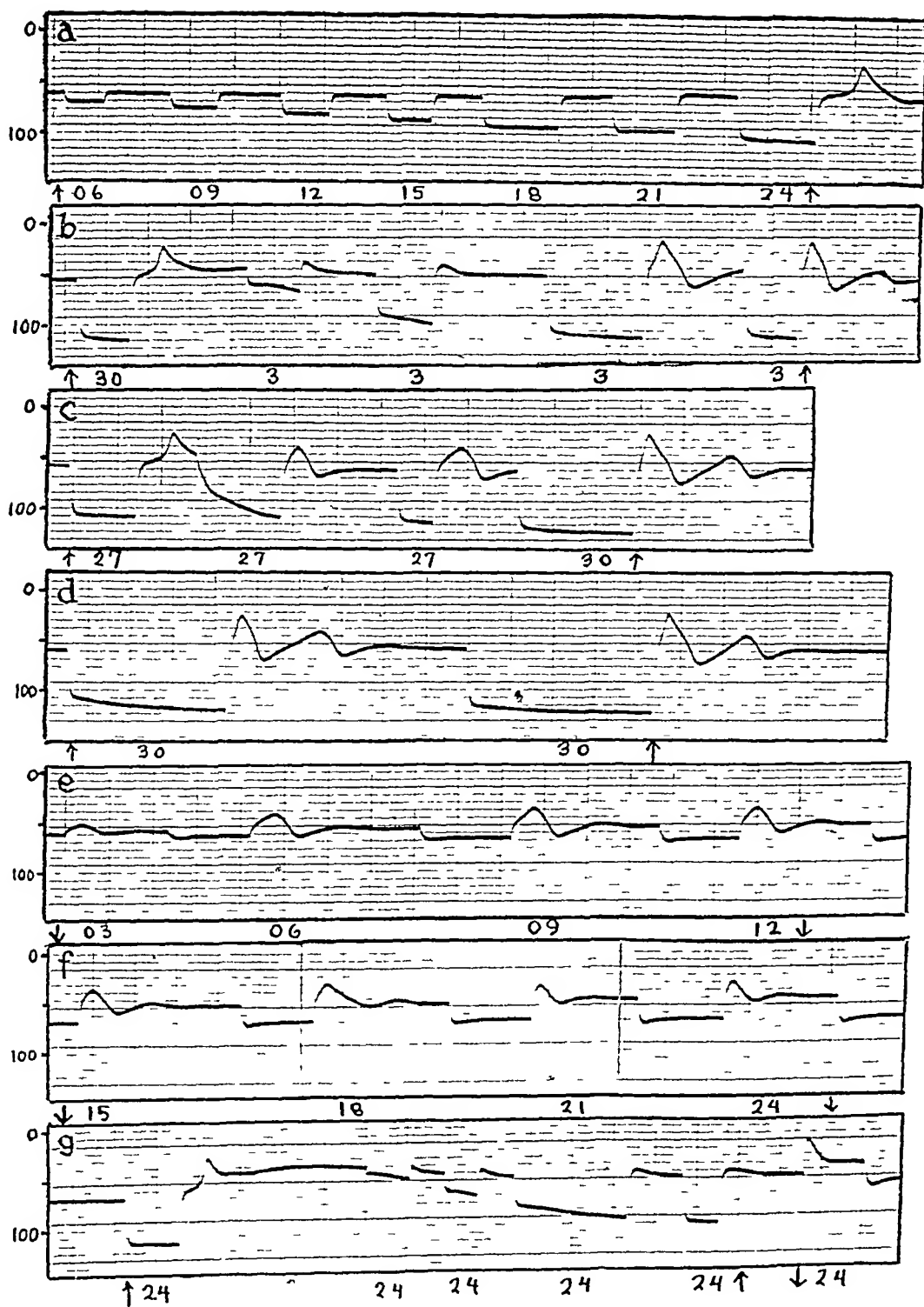tremely large outward currents do not produce typical stimulation,

Fig 8

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only in irregular polarization, with cusps (Figs 6 and 7)   This may
occur more and more as the result of repeated stimulation (Fig 6), and
probably is correlated with the low positive P D reached in this condi
tion, so that action currents of normal magnitude become impossible
It is therefore to be distinguished from those cells reported by Oster
hout and Hill,[6] which are either naturally or experimentally not capa
ble of stimulation, yet which may be rendered sensitive by treatment
(Such cells have a high positive P D)

In contrast to the behavior of nerve, there appears to be no hyper
sensitive period in *Nitella* during which excitability is heightened,
following an action current

Special Effects of Inward Currents

These include in addition to the regular polarizations considered
above, stimulation at break, and restoration of positive potentials

Break Effects —It was mentioned above that at the break of inward
currents, stimulation sometimes occurred   Examples are shown in
Fig 6   There is nothing startling about this, which should be ex
pected as a result of a temporarily greater residual outward current,
due to discharge of the heightened positive potential produced by
inward current   It would be expected in those cells with the lowest
threshold for stimulation by outward currents, and this was generally
found to be true   Fig 6 also shows again that recovery of polariza
bility after stimulation occurs for inward as well as outward currents
indeed it may be aided by such current flows   Before taking up this
restorative function, two other points may be noted   One is that a
second stimulation does not usually follow on breaks of successive

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FIG 8 Records showing polarization and partial stimulation by current flow
in *Nitella*   Particularly to be noted the quick spike like response either to
break of inward current or during outward flow   followed by rapid recovery   a
second upward movement and recovery   and even a third the series dying away in
decremental fashion like a damped oscillation   NaCl solutions tend to produce
this effect

Currents in $\mu a/cm$ direction being indicated by arrows   inward current upward
and outward current downward   Sensitivity about 7 mv per horizontal division
the P D being that of one contact only (the other contact chloroformed)   Time
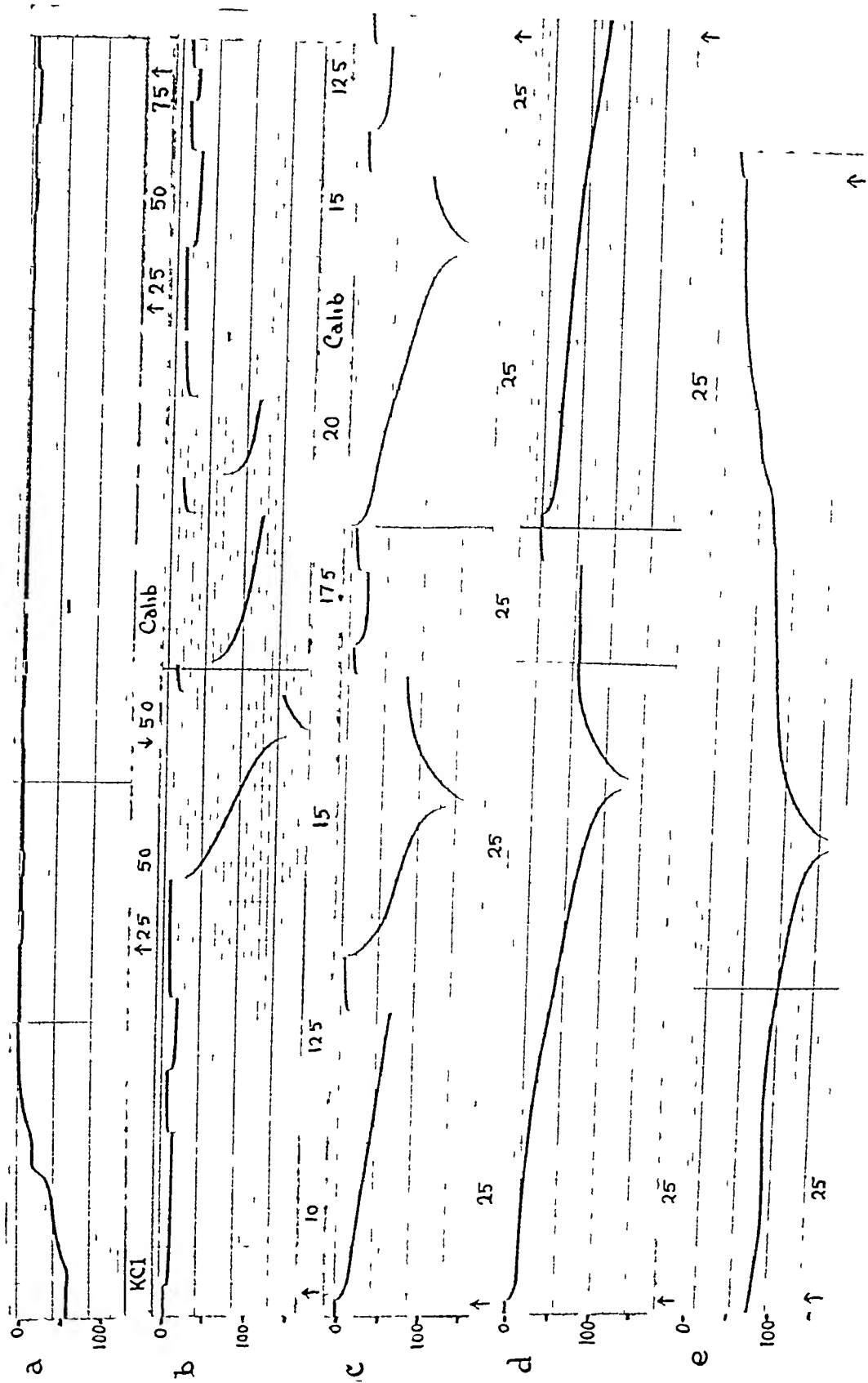marks 1 second apart

Fig 9

later inward currents of the density originally effective (Fig 6), show ing an increase of threshold analogous to that for outward currents This does not always hold, however, as shown in Fig 7, where it appears that large outward flows may sensitize the cell to break stimu lations to a greater degree than to normal outward flow   A second point is that stimulation at break may be incomplete, just as at make, the depolarization curve beginning to "overshoot" beyond the original P D line, but recovering without complete stimulation   Examples are shown in Fig 6   This is probably due to too short a flow of the aug mented residual current which falls to a subthreshold value before stimulation is thoroughly initiated

Sometimes a second or even a third partial stimulation of this sort occurs, either during outward flow, or at the break of inward current making a decremental ("die away") series as shown in Fig 8   There is no fully satisfactory explanation of these "damped oscillations' which probably represent the interaction of the stimulated and nearby regions, without a true action current occurring   Osterhout has recently suggested that they represent effects at only one (probably the inner) surface, the circuit being completed through the proto plasmic interior without the participation of the other (outer) surface [11]

FIG 9 Effects of current flow on *Nitella* cell exposed to KCl   The cell was previously chloroformed at one contact   0 05 M KCl was then applied at the intact end giving the decrease (and slight reversal) of P D shown in Record *a*   Currents were then passed as shown densities up to 15 $\mu a/cm^2$ producing only slight counter E M F   At 15 $\mu a/cm$ inward current however a large polarization results with sigmoid course and a marked rise to a sharp cusp followed by a recession   On cessation of current flow the counter E M F drops away abruptly   Larger densi ties now produce more rapid polarizations which however do not rise to appre ciably higher values and do not show the cusp   After these flows a return to 15 $\mu a/cm$ is ineffective only at 25 $\mu a/cm$ does a good polarization again appear and here again a third flow is ineffective (Record *c*)   These curious alternations of response are probably due to breakdown after the cusps which requires some time for repair   in fact finally as in Record *c* the polarization may entirely die away during the flow of current

Current densities in $\mu a/cm$ as marked   inward flows being designated by upward arrows   outward current (only once in *a*) by a downward arrow   Sensi tivity about 8 mv per horizontal division as marked on each record and shown by calibrations of 50 mv in Records *a* and *b*   Time marks 1 second apart

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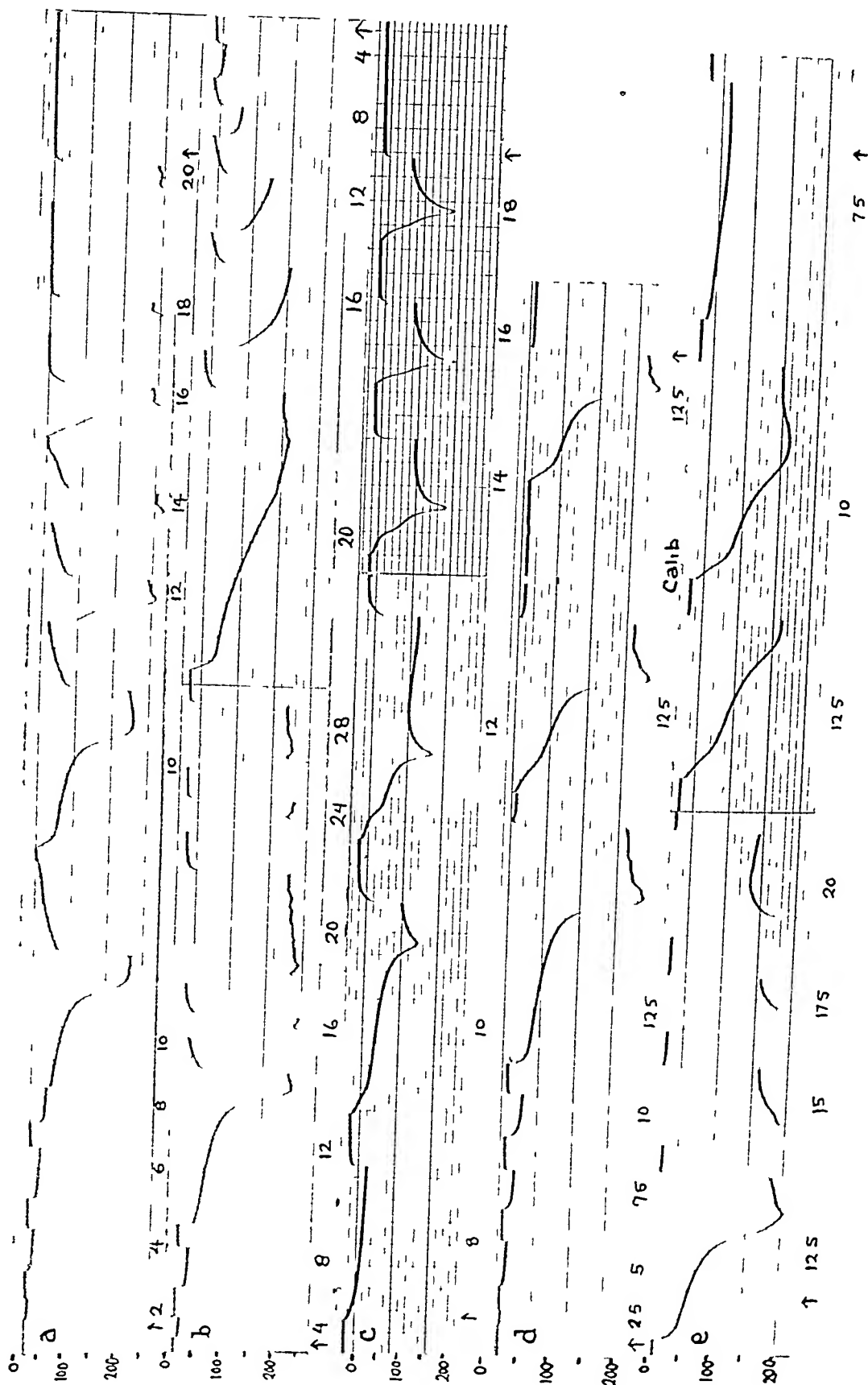[11] Osterhout W J V and Hill S E *J Gen Physiol* 1934-35 **18**, 499

Fig 10

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Somewhat analogous rhythmic effects have been found in *Halicystis*
so far unpublished there they suggest a delayed response (possibly
metabolic) to the stimulus resulting in temporary overcompensation,
followed by recovery, then a renewed stimulation, etc

Restorative Effects —It was mentioned above that recovery of P D
after stimulation may be speeded up by a continued or even an inter
rupted, flow of current inward across the protoplasm Examples
suggesting this are included in Fig 5, although the normal recovery
varies sufficiently in speed to make it somewhat uncertain

More striking evidence of the restorative action of inward currents
comes from experiments with cells which have been exposed to a
threshold concentration of KCl (0 01 M or higher) Reasons have
been previously given[1] for regarding such cells as permanently stimu
lated, since the KCl appears to produce its more profound effects
largely by inhibiting recovery from stimulation (whether induced
electrically, mechanically, or as often happens, by the KCl itself,
either immediately on application, or after a delay) The P D now
remains permanently depressed (or even reversed) and the cells are

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Fic 10 Records showing further characteristics of response to inward current
under KCl treatment The *Nitella* cell has previously been chloroformed at one
end and exposed to 0 05 M KCl at the recorded end Inward current is then
passed Small though regular polarizations are evoked by currents up to 8μA/cm ^{2}
At 10 μa/cm a sigmoid polarization produces over 200 mv positive P D A repe
tition of this produces a slightly faster response while higher currents greatly
accelerate the time course which becomes very abrupt at 20 μa/cm with
out however greatly increasing the positive P D reached Record *b* shows a
second series of inward currents at first increasing in density then decreasing
c is a record of another cell similarly treated and *d* and *e* of a third cell showing
the general similarity of responses In *e* note particularly that the response to
successive flows of 12 5 μa/cm becomes somewhat faster and that to higher densi
ties much faster but the positive P D reached is somewhat less apparently due to
an injury following rapidly on the sharp cusp

Sensitivity about 13 mv per horizontal division (50 mv calibration in *d*)
Time marks 1 second apart Current densities in μa/cm all inward (as indi
cated by upward arrows)

The resemblance of these curves to those taken with *Valonia* previously pub
lished[3] is striking

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[1] Blinks L R *Proc Soc Exp Biol and Med* 1932-33 **30**, 756

practically non-reactive to currents of the magnitudes ordinarily evok-
ing polarizations (e g up to 5 $\mu a/cm^2$) The effective resistance
consequently falls nearly (though not quite) to that of dead cells (cf
footnote 1) However, if sufficiently large currents be passed inward
(e g , about 10 $\mu a/cm^2$) polarizations begin to appear, at first with a
very slow rise, an inflection, and then a rapid rise to high positive values
(100 to 200 mv) Examples are shown in Figs 9 and 10 This large
P D drops off again to low values almost instantly on the cessation
of current flow Successive inward currents of the same density
produce the effects more rapidly, as do greater densities, they do not,
however, greatly increase the magnitude of total change, indeed, the
higher densities may produce smaller P D 's, probably due to injury
The sharp cusp almost invariably appearing with larger currents is
probably to be explained as a recession due to injury following the
abrupt rise Return to smaller densities sometimes produces polariza-
tions which were lacking before the larger current flow, causing a
hysteresis much like that in *Valonia*[3] and *Halicystis*,[4] but there is
always a point at which polarization again disappears—again a real
threshold effect

That this striking behavior in the presence of KCl is not due to
permanent injury is shown by the almost immediate restoration of
normal polarizations, and eventually of stimulations, on re-exposure
of the cells to NaCl or tap water On the other hand polarizations
are entirely lacking, even to very large inward current densities, when
the cells are chloroformed

Practically no polarizations occur to outward currents in the pres-
ence of KCl, even at very high current densities This is very like
the situation in "variable" *Valonia*[3] (except that in the latter, polar-
izations due to temporary "conditioning" by previous inward flow
may occur) In *Valonia*, however, KCl does not influence the be-
havior

COMPARISONS AND DISCUSSION

Several comparisons have already been made between the behavior
of *Nitella* and that of *Valonia* and *Halicystis* On the whole, the same
phenomena may be found in each, although the conditions vary con-
siderably Regular counter E M F 's (polarizations) occur in all, being

most marked in *Nitella*, less pronounced in *Halicystis* (especially in impaled cells, where they are very slow, and rather small in magnitude), and almost entirely absent in the variable state of *Valonia*, where they appear only on recovery from injury, or by specific treatments   They disappear in *Nitella* during stimulation, and as long as recovery is inhibited by KCl, they are suppressed in *Valonia* by exposure to ammonia in sufficient concentration, while in *Halicystis* they persist, or become even greater and faster, during reversal of P D by ammonia, etc   They may be restored in *Nitella* and *Valonia* by the passage of sufficiently large inward currents, *which also restore the positive* P D

On the other hand, the positive P.D of both *Nitella* and *Halicystis* is either destroyed or reversed by the passage of sufficient outward current, with definite threshold effects in each case, and very similar time curves   Differences occur in recovery, this process in *Nitella* being powerful enough to occur in spite of a continued flow of outward current which had initiated stimulation, while in *Halicystis*, recovery occurs only on cessation or considerable reduction of the original outward current flow   (In the presence of critical concentrations of ammonia, however, its P.D may remain permanently reversed after cessation of outward current)

Another common characteristic is the thresholds, which tend to *carry the potentials from one level abruptly to another without intermediate values*   These are found not only in the stimulation of *Nitella*, the reversal of potential in *Halicystis*, and in the production of positive potentials in *Valonia*, by current flow, but also in the potential changes in KCl treated *Nitella*, and ammonia treated *Halicystis* There is an all or none character to these potential levels, about which, as a base, the more regular counter E M F 's build up (when present) Such levels occur near zero and at 100 mv positive or more in *Nitella*, at some 50–60 mv positive and 20–30 mv negative in *Halicystis*, and at 8 or 10 mv negative and some 50–100 mv positive in *Valonia* Very seldom are intermediate levels found, if the potential is carried by current flow or otherwise to these values, it usually goes beyond them to the other level, where it is again more stable

How may these facts be fitted into one picture? The simplest assumption is that current flow produces two effects, the first, regular

counter E M F 's, dependent upon the presence of some structure (sur-
face film or membrane) but not altering it, the second, a direct altera-
tive effect upon the structure itself, either destructive or restorative
For the present, it is unimportant whether the counter E M F is due
to a polarization, dependent on the differential mobility of two species
of ions, or to a static capacity resulting from impermeability (or equal
permeability) to all ions   It is reasonable to suppose, however, that
polarization is involved, since the bioelectric potential itself probably
depends upon a differential mobility of ions, and we have seen that on
the whole counter E M F 's occur when there is also a large, usually
positive P D displayed across the protoplasm   Current flows of
moderate density would presumably also produce counter E M F 's by
setting up across the surface a new concentration gradient of ions
either of those normally responsible for the bioelectric potential itself,
or of any others having appreciable mobility in that surface

Even up to considerable inward currents, such polarizations are all
that occur, although the counter E M F is finally limited, either by
establishment of a maximum ionic gradient (which would be some
million-fold, to give a potential of 300 to 400 mv), or by "breakdown"
of the surface film   (We might here picture the opening of minute
holes or pores, which promptly repair on cessation of current, since
the injury due even to rather large inward current is not great)   In-
deed the contrary holds for we have seen that inward currents have a
strong restorative effect, producing positive P D and polarizability
when these are lacking, as in *Valonia* or KCl-stimulated *Nitella*

Outward currents, on the other hand, produce much less counter
E M F before the more drastic changes of stimulation or potential
reversal come into action   Here we must postulate no minor break-
down but a profound alterative effect on the surface film, causing its
temporary disappearance or non-functioning, either with loss of both
P D and polarizability in *Nitella*, or of positive P D in *Halicystis* (where
a second, non-affected surface maintains the negative potential and
continued polarizability)   Only in *Valonia*, where the P D is already
negative, and polarizations are either absent or small, does outward
current produce no further effect, we may assume this is because such
Valonia cells are already "permanently stimulated "   When polariza-
bility has been temporarily restored by inward currents, however, it

is destroyed again by outward currents,[3] more rapidly than it dis appears spontaneously

Mechanism of Current Flow Effects

What can cause this destructive action of outward currents, and the restorative effects of inward ones? "Dielectric breakdown" ought to occur equally well with inward as with outward currents Differ ential concentration effects due to ionic movement and changes of ionic gradients can be expected to occur however These might include (a) hydrogen (or hydroxyl) ions, (b) other inorganic ions, especially potassium, (c) organic ions of the protoplasm (including constituents of the surface film itself) These will be discussed in order

Hydrogen Ion —A change of acidity with current flow across mem branes or other surfaces has been postulated at least since the experiments of Bethe,[13] who definitely found it to occur with various models, and believed he demonstrated it in living plant cells The latter has been called into question by the present author, who found that the more obvious of the color effects in living cells containing a natural indicator, was due to migration of the latter, rather than to a change of acidity [14] This does not preclude, however, a change of acidity in addition, in a layer too thin to be seen, adjacent to the *protoplasm, such is in fact all that might be expected, and all that* would be necessary to produce the effects on the surface itself Here, as often, the wrong experiment may have pointed to the right conclu sion For there is much indirect evidence to support the hypothesis of acidity changes due to current flow Some of this has been dis cussed in the papers on *Valonia*[3] and *Halicystis*,[4] where it was shown that not only by the similarity of their effects, but actually by their combination in the same experiment, outward currents resembled and assisted treatments with ammonia and other penetrating bases, while

[13] Bethe H *Arch ges Physiol* 1916 163, 147

[14] Blinks L R *Proc Soc Exp Biol and Med* 1931–32, 29, 1186 The results given here have been recently confirmed in the author s laboratory on still more favorable material by Mr R D Rhodes, who has obtained objective photographic and spectrographic evidence of the effects This is shortly to be arranged for publication

inward ones opposed their effects, resembling and assisting treatments with dilute acids, and certain very active though weakly acidic substances like phenol, cresol, etc [3] So far, there is not much evidence of this sort available for *Nitella,* but a similar situation may be postulated

There remain two questions concerning the physical and chemical mechanism of such acidity changes   Is it justified on the basis of known ionic mobilities?   And what effect could acidity changes have on the surface?   Concerning the first, there is not very positive evidence, for pH changes are in most cases rather ineffective in altering the P D   Only in the case of *Halicystis,* and there in only one species,[15] is there evidence of a high mobility of hydrogen ion in the protoplasm The general absence of its effect may, however, be due to the necessarily low concentrations of H ion which can be safely applied to cells, in comparison with the other ionic concentrations present

Granted that the mobility of H ion might give rise to acidity changes, it is quite reasonable to postulate effects of such changes on the surface through, for example, the influence of pH upon the ionization of its constituents, whether these were ampholytes such as proteins, or weak acids such as fatty acids   It has been mentioned before that the sharply critical all-or-none levels in the cells scarcely coincide with the long smooth dissociation curves of proteins, which show no very abrupt and complete change of sign with a small pH shift   More in keeping with various facts is a lipoid layer, which gives rise to the observed electrical effects (P D , polarization) while intact, but ceases to function when interrupted or removed   A monomolecular arrangement might contribute to the all-or-none nature of its disruption but this is not necessary, since the effects of a thicker layer would probably also persist until it was disrupted, the latter occurring by saponification when alkalinity was sufficiently high   That an inner layer, or the inner side of an outer layer is involved seems indicated by the action of ammonia which apparently penetrates as NH_3 across the outer surface to reach the sensitive region on the inner side, while other alkalies such as sodium and potassium are less effective, because

[15] Blinks, L R , *J Gen Physiol* , 1933–34, 17, 109

they do not penetrate as readily   (When potassium is once admitted, however, as by stimulation of *Nitella*, it appears to be very tenacious in its effects [12])

Restoration of the film might occur by increased acidity, whether produced by current flow inward, or by metabolic processes   The latter apparently come spontaneously into prompt and powerful play immediately after stimulation in *Nitella* so that recovery occurs in spite of a continued outward current flow of a density which caused the original stimulation   They are less powerful in *Halicystis*, where recovery occurs only on cessation or diminution of outward current And, finally, they are too weak even to maintain the surface, in the variable state of *Valonia*, in *Nitella* treated with KCl, or in *Halicystis* treated with ammonia, unless assisted in each case by inward current

Other Inorganic Ions —Potassium, by virtue of its usual apparently high mobility in the protoplasm, would presumably be moved from the sap across the surface by outward currents, to reduce both P D and resistance when its concentration on the outer side of the surfaces approached that in the sap   This has been made the basis of a theory of the action current by Osterhout [16]   There are, however, several objections to this   (1) Why should stimulation, once initiated, go on to completion even though current flow ceases (Figs 3, 4, 5)?   (2) Why should the cells recover from stimulation even though the stimulating current continues to flow, presumably still carrying out potassium ions from the sap across the protoplasm?   (3) Why should inward currents have a restorative effect in the presence of KCl, since here potassium ions would be brought continuously into contact with the outer surface?   (Similar restoration also occurs in the presence of KCl with *Valonia*)

Nevertheless, it is perfectly clear that potassium produces profound electrical effects in *Nitella*, which are aided and duplicated by outward currents and are opposed and counteracted by inward currents If they are not due to the high mobility of the K ion, then to what? The author has suggested [12] that this may also be through an acidity effect within the protoplasm, potassium entering the cell either as

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[16] Osterhout, W J V *J Gen Physiol* , 1934–35, **18**, 215

KOH, by formation of an organic salt KX, or by exchange of K for H ions, but in any case increasing the alkalinity in some critical region   The surface film is thereby altered or destroyed and recovery from stimulation is inhibited (unless an inward current is passed) Too great emphasis is not placed upon this theory, which is supported mostly by analogy with the ammonia effects in *Halicystis*[16], in view of recent experiments by Jacques and Osterhout,[17] and by Collander[18] it may be questioned whether potassium enters *Nitella* in this manner

Organic Ions —Another aspect has been recently given to the potassium effects by the experiments of Osterhout and Hill[19] which show that stimulation as well as potassium effects can be abolished by various treatments, such as long exposure to distilled water, and again restored by various substances   This they explain by the leaching out of a substance *"R"* from the cell surface   It is possible that current flow acts upon R, moving it from the cell interior to the surface during outward current flow, or back from the surface into the interior during inward flow, depleting it in the surface, and so causing loss of the potassium effects   Whether R is therefore a cation, and acts by sensitizing the surface in some way to potassium, remains to be seen

The simplest hypothesis of all, would be that the surface itself is composed of an ionic species, capable of migrating in an electric current   If these ions had a negative charge (*e g* were fatty acid radicals) they would be attracted to the outer side of the protoplasm by the flow of positive current inward, or driven back from the surface into the protoplasm by outward currents   The observed threshold might represent the point at which the applied electrical gradient just overcomes the forces holding the ions in the surface (surface activity, or chemical affinity for the protoplasmic matrix)

No choice can yet be made between these several hypotheses of the mechanism of current flow effects, which are suggested for future testing   On the whole, there is the most evidence in favor of a change of acidity, but this does not preclude other effects, or a combination of them

[17] Jacques, A G , and Osterhout, W J V , *J Gen Physiol* , 1934–35, **18**, 967
[18] Collander, R , abstract in *Proc 6th Int Bot Cong* , *Amsterdam*, 1935, **2**, 289
[19] Osterhout, W J. V , and Hill, S E , *J Gen Physiol* , 1933–34, **17**, 87, 99, 105

Comparisons with Animal Tissues —The results with *Nitella* are in agreement with the work of McClendon[20] and others [1] who have found a decrease of effective resistance to accompany the stimulation of muscle On the other hand Bozler[22] has recently found an increase of impedance during the stimulation and contraction (isometric) of frog sartorius muscle It is possible that the latter result finds a parallel in the behavior of *Halicystis* In this organism the passage of out ward current produces a reversal of P D bearing much resemblance to the *Nitella* stimulation curve During the course of reversal, small increments or decrements of this current have a much larger effect upon the P.D than they do at either fully positive or fully negative values [4] The same is true of *Valonia* during restoration of the positive potential by inward currents [3] Since the changes of P.D are always in the direction of a counter E.M.F (opposing the flow of current) they would have the effect of an increased impedance or effective resistance, and could not be dis tinguished from such, even though they might be rather due as here suggested to the extreme lability of the cell surface or other structure responsible for the P D, which was being destroyed or reconstituted by the flow of current It may be that the muscle under proper conditions of stimulation shows a similar lability and sensitivity to current flow which was reflected in Bozler's measurements

SUMMARY

String galvanometer records show the effect of current flow upon the bioelectric potential of *Nitella* cells Three classes of effects are distinguished

1 Counter E.M F's, due either to static or polarization capacity, probably the latter These account for the high effective resistance *of the cells They record as symmetrical charge and discharge curves,* which are similar for currents passing inward or outward across the protoplasm, and increase in magnitude with increasing current density The normal positive bioelectric potential may be increased by inward currents some 100 or 200 mv, or to a total of 300 to 400 mv The regular decrease with outward current flow is much less (40 to 50 mv) since larger outward currents produce the next characteristic effect

2 Stimulation This occurs with outward currents of a density which varies somewhat from cell to cell, but is often between 1 and 2 $\mu a/cm^2$ of cell surface At this threshold a regular counter E M F

[20] McClendon J F *Am J Physiol* 1912, 29, 302 1929 91, 83 *Protoplasma* 1929 7, 561

[21] *Cf* especially the recent work of Dubuisson M *Arch Int Physiol* 1933 37, 35, 1934 38, 85, 460, 468, 1935, 41, 177 511

[22] Bozler E, *J Cell and Comp Physiol*, 1935 6, 217

starts to develop but passes over with an inflection into a rapid decrease or even disappearance of positive P D , in a sigmoid curve with a cusp near its apex   If the current is stopped early in the curve regular depolarization occurs, but if continued a little longer beyond the first inflection, stimulation goes on to completion even though the current is then stopped   This is the "action current" or negative variation which is self propagated down the cell

During the most profound depression of P D in stimulation, current flow produces little or no counter E M F , the resistance of the cell being purely ohmic and very low   Then as the P D begins to recover, after a second or two, counter E M F also reappears, both becoming nearly normal in 10 or 15 seconds   The threshold for further stimulation remains enhanced for some time, successively larger current densities being needed to stimulate after each action current   The recovery process is also powerful enough to occur even though the original stimulating outward current continues to flow during the entire negative variation, recovery is slightly slower in this case however

Stimulation may be produced at the break of large inward currents, doubtless by discharge of the enhanced positive P D (polarization)

3 Restorative Effects —The flow of inward current during a negative variation somewhat speeds up recovery   This effect is still more strikingly shown in cells exposed to KCl solutions, which may be regarded as causing "permanent stimulation" by inhibiting recovery from a negative variation   Small currents in either direction now produce no counter E M F , so that the effective resistance of the cells is very low   With inward currents at a threshold density of some 10 to 20 μa/cm ^{2}, however, there is a counter E M F produced, which builds up in a sigmoid curve to some 100 to 200 mv positive P D This usually shows a marked cusp and then fluctuates irregularly during current flow, falling off abruptly when the current is stopped Further increases of current density produce this P D more rapidly, while decreased densities again cease to be effective below a certain threshold

The effects in *Nitella* are compared with those in *Valonia* and *Halicystis*, which display many of the same phenomena under proper conditions   It is suggested that the regular counter E M F 's (polarizations) are due to the presence of an intact surface film or other struc-

ture offering differential hindrance to ionic passage   Small currents do not affect this structure, but it is possibly altered or destroyed by large outward currents, restored by large inward currents   Mecha nisms which might accomplish the destruction and restoration are discussed   These include changes of acidity by differential migration of H ion (membrane "electrolysis"), movement of inorganic ions such as potassium, movement of organic ions, (such as Osterhout's sub stance R), or the radicals (such as fatty acid) of the surface film itself Although no decision can be yet made between these, much evidence indicates that inward currents increase acidity in some critical part of the protoplasm, while outward ones decrease acidity

THE EFFECT OF HEMOLYTIC SUBSTANCES ON WHITE CELL RESPIRATION

By ERIC PONDER and JOHN MACLEOD

(From The Biological Laboratory, Cold Spring Harbor Long Island)

Substances such as saponin, the bile salts, the soaps, etc, which are generally classified as hemolysins, have a much wider cytolytic effect than is usually recognized   The purpose of this paper is to show that these hemolytic substances affect white cells in much the same way as they affect red cells, the arbitrary criterion of their cytolytic effect being their ability to depress white cell respiration

Preparation of Material

The white cell suspensions were prepared from rabbit peritoneal exudates, obtained by a method similar to that of Mudd Lucké McCutcheon, and Strumia (1929)   Some 18 hours before the suspension is required each rabbit receives about 300 cc of sterile 0 95 per cent NaCl intraperitoneally the resulting exudate is very rich in leucocytes, and can be drawn off without any further introduction of NaCl and without the use of citrate   About 0 25 cc of leucocytes is obtained for each 15 cc of exudate and at least 95 per cent of the cells are polymorphs

The exudate is transferred to centrifuge tubes, and spun at the rate of about 1000 R P M for 2 minutes the supernatant fluid is then replaced by buffered NaCl in sufficient quantity to make a suspension containing from 60,000 to 100,000 cells per mm[3][1]   The exact number present is found by making a count in the usual manner   Barron and Harrop (1929) have found very irregular results for the respiration of leucocytes from rabbit peritoneal exudates, and have attributed the irregularity to centrifuging, Fujita (1928) is of the same opinion, and also says that changes of temperature have a similar harmful effect on the respiration   Our results for white cell respiration, on the other hand are very regular and consistent from one exudate to the other nor does the cell respiration appear to be appreciably

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[1] This buffered NaCl is prepared by adding 25 cc. of a mixed phosphate buffer (72 cc M/15 Na$_2$HPO$_4$ and 28 cc M/15 KH$_2$PO$_4$) to 75 cc of 1 2 per cent NaCl The resulting solution is isotonic for the rabbit and has a pH of 7 1   Mammalian Ringer s solution (Bayliss) can be used instead of this buffered NaCl, but without any appreciable advantage

affected by successive centrifuging, even if the cells are spun as many as three times at high speeds   At the same time, our results do not confirm Fujita's in showing that changes in temperature produce a harmful effect, for suspensions of cells used directly after preparation do not show any greater respiration than do suspensions kept in the ice box for intervals up to 12 hours

In the case of the respiration of leucocytes in whole blood, Barron and Harrop (1929) and Soffer and Wintrobe (1932), have pointed out that cell concentration has a marked influence on metabolic activity, the O_2 consumption varying roughly inversely with the concentration, it is therefore necessary to find the effect of (a) cell number, and (b) the "crowding" of cells, on the O_2 consumption in the suspensions prepared from peritoneal exudates   The effect of cell number is shown in Table I, in which the number of cells per mm³ is shown in the first column, the O_2 consumption in mm³/hr in the second, and the ratio of the number to the O_2 consumption in the third   In these experiments, the cells were contained in 5 cc of saline

TABLE I

Cells/mm ³ — 10³	O_2	Ratio
	mm ³/hr	
125	136	1 09
75	80	1 07
37 5	42	1 12
18 75	20	1 07

The figures in the last column are substantially constant, and so the O_2 consumption is very nearly proportional to the number of cells within the experimental range   Cell suspensions containing more than about 125,000 cells per mm³ are very viscous, and homogeneous samples are almost impossible to obtain because of agglutination   The effect of crowding the cells within this concentration range can be dismissed briefly, for we have found that it is immaterial whether one suspends a given number of cells in 5 cc of fluid or in a volume twice as great Speaking generally, the effect of cell concentration appears to be very different in the case of leucocytes in these suspensions from what it is in the case of the leucocytes of whole blood (cf Barron and Harrop (1929), Soffer and Wintrobe (1932))

Respirometry

The measurements of O_2 consumption were made in Fenn respirometers with 35 cm capillaries of about 0 9 mm bore, and with cups of capacity about 25 cc The respirometers were contained in a water bath at 37 5°C , controlled to within 0 01°C , and were rocked to and fro through an arc of 70° at the rate of 100 per minute

In order to investigate the effect of lysins such as saponin, the bile salts etc, on O consumption of white cells, 2 cc. of a suspension containing about 70 000 cells per mm^3 are placed in one of the cups of the respirometer, and in the other cup is placed the same volume of saline   In both side cups is placed 1 cc of the lysin in the concentration desired, so that on tipping the respirometer the lysin can be added to the cell suspension and the balancing volume of saline respectively   The respirometer is equilibrated in the water bath for 30 minutes   at the end of which time the O$_2$ consumption of the suspension is measured at 10 minute inter vals for a second period of 30 minutes   The respirometer is then tipped so that the lysin is added to the cells and the O$_2$ consumption measured at 10 minute intervals for 110 minutes   this is in some respects, an arbitrarily selected time but after the cells have been in the respirometer at 37°C for more than about 3 hours their rate of O$_2$ consumption is apt to decrease spontaneously even in the absence of lysin

In experiments on the effect of hypotonicity on white cell respiration the proce dure was slightly modified   It is technically unsatisfactory to add volumes of water from a side cup in order to produce various degrees of hypotonicity, and so the cells were suspended in saline of various degrees of hypotonicity before being introduced into the respirometers   After 30 minutes for equilibration, readings of the O$_2$ consumption were made for a further 30 minutes   The O$_2$ consumptions during this period in the case of each hypotonic system are expressed as a per centage of the O$_2$ consumption in a system made up with isotonic saline

The Normal O$_2$ Consumption of the White Cells

When suspensions of leucocytes are obtained in the manner de scribed above, the irregularities in O$_2$ consumption described by Barron and Harrop (1929) are not observed, in fact, the O$_2$ consump tion is remarkably constant from one exudate to another, and the rate of O$_2$ consumption is very constant over a period of hours   Our Q_{O_2}'s vary between -4 5 and -7 0, with a mean value of -5 5, which is considerably lower than the value of -9 0, given by Fujita (1928) for rat leucocytes obtained from blood   Calculating Barron and Harrop's constant K, we find a mean value of 0 7, which compares very well with their value of 0 8 found for the white cells of leukemic blood, it is doubtful, however, if a comparison can properly be made

The Effect of Lysins on the O$_2$ Consumption

1 Saponin —The typical effect of saponin (and, probably, of most lysins) on white cell respiration is shown in Fig 1, in which 1 cc of 1 in 1000 saponin is added to 2 cc of the white cell suspension (68,000

cells per mm³)   The O₂ consumption over any 10 minute period is expressed as a percentage of the average O₂ consumption per 10 minute period found before the addition of the lysin at the point marked in the figure   Since the tipping of the respirometer itself causes a movement of the drop in the capillary, 5 minutes are allowed for equilibration, and readings are made every 10 minutes thereafter

This typical curve shows the following characteristics   (a) The addition of the lysin is followed by an increase in O₂ consumption which lasts about 20 minutes   This increase is not due to a specific effect of the lysin, for it can be obtained in control experiments in which
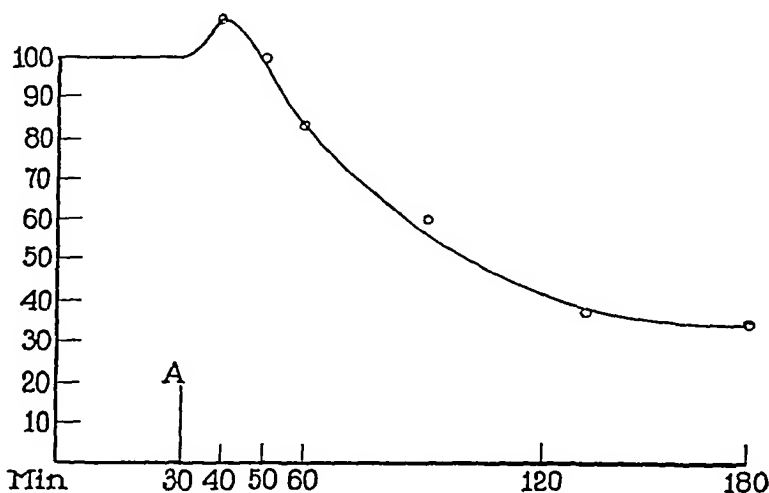
FIG 1 Ordinate, O₂ consumption as a percentage of that occurring before the addition of the lysin, which was added at point marked A   Abscissa, time in minutes

Ringer's solution only is added to the cell suspension, in such experiments, indeed, the increase is of much longer duration   (b) The curve then falls off roughly exponentially towards a new level, which it reaches about 100 minutes after the addition of the lysin   When large amounts of lysin are added, this new level may correspond to only some 5 per cent of the initial respiration, under which circumstances it is reached relatively quickly and is steadily maintained, when smaller quantities of lysin are added, on the other hand, the new level is reached more slowly and may correspond to quite a considerable fraction of the initial respiration, under which circumstances

it is generally not steadily maintained, principally because white cell respiration tends to decrease spontaneously after about 3 hours at 37°C

These points are well shown in Fig 2, in which curves are plotted for dilutions of saponin from 1 in 300 to 1 in 1250 It will be noticed that there is no temporary increase in O_2 consumption corresponding to the addition of large quantities of saponin (1 in 300 and 1 in 500), any increase which occurs being masked by the almost immediate decrease which follows the addition of the lysin A curve showing the effect of the addition of Ringer only is given for comparison
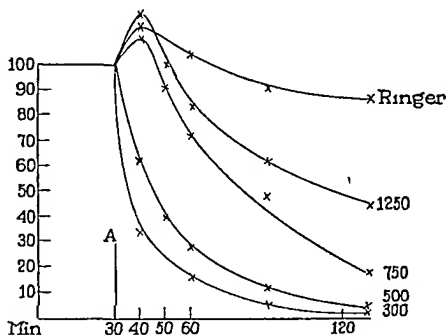
FIG 2 Ordinate and abscissa as in Fig 1   The figures opposite each curve show the dilution of saponin added at the point marked A In the case of the curve marked "Ringer", Ringer s solution only was added

Using the same data as that from which Fig 2 was constructed, we can tabulate the percentage of the original O_2 consumption remaining 110 minutes after the addition of the saponin against the quantity of saponin added, and the result is shown in Table II As has already been remarked, the period of 110 minutes is in some respects an arbi trarily selected time, it is, however, as long a time as we can allow, spontaneous decreases in respiration being taken into consideration For the purpose of interpreting the results, we shall take it that after 110 minutes of action of the saponin, a new steady state has been reached

These results are plotted in Fig 3, with the residual respiration as the ordinate   The curve presents several features   (a) When 0 5 mg of lysin or less is added to the system, there is no observable effect on the O₂ consumption   This quantity of saponin is very much greater than that required to hemolyze red cells under comparable

TABLE II

Dilution of saponin, 1 in	Saponin added	Residual respiration
	mg	*per cent*
300	3 33	2
500	2 00	4
750	1 33	18
1000	1 00	38
1250	0 80	44
1500	0 67	54
2000	0 50	100
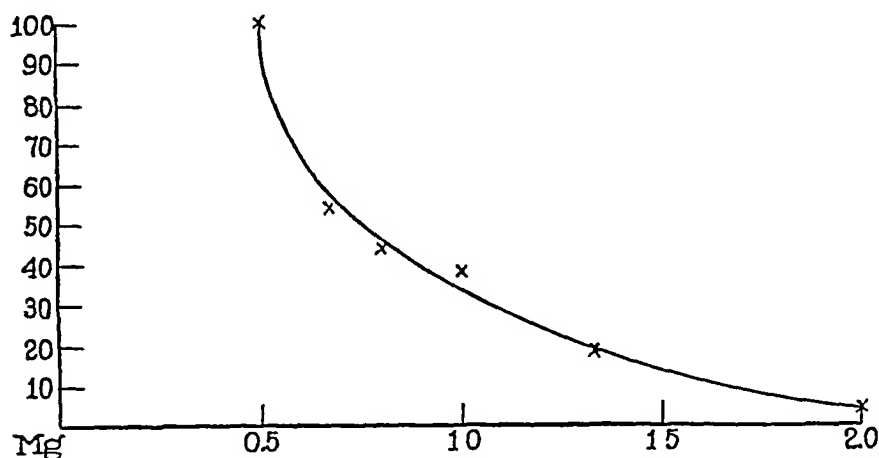
FIG 3 Ordinate, residual respiration 110 minutes after the addition of the lysin, abscissa, quantity of saponin added, in milligrams

conditions, thus to produce 50 per cent diminution in the O₂ consumption of the white cells, we require about 0 7 mg , or 700 microgm , of saponin, whereas to produce 50 per cent lysis of the same number of red cells only about 20 microgm would be needed   This is not remarkable, for lysis of the red cell is an effect on a surface layer, whereas

the material involved in white cell respiration is no doubt distributed throughout the cell volume   (b) It is difficult to decide whether or not a sufficiently great amount of saponin would abolish the O_2 consumption entirely, and the point is not of much importance, the addition of 10 mg of the lysin, however, results in the residual respiration being less than 1 per cent of the initial   (c) The most interesting point about the curve is that a small increase in the amount of saponin above 0 5 mg produces a relatively very great effect, a factor of 3 0, indeed, in the amount of saponin added makes the difference between 100 per cent and 20 per cent residual respiration   A similar situation is met with in the case of the hemolysis of red cells, where an increase in the concentration of lysin by a factor of 3 0 makes the difference between just commencing hemolysis and about 80 per cent lysis

TABLE III

Sodium taurocholate		Sodium glycocholate	
Lysin added	Respiration	Lysin added	Respiration
mg	*per cent*	*mg*	*per cent*
10 0	30	10 0	25
5 0	51	5 0	50
3 3	77	3 3	72
2 5	100	2 5	95

(d) In the case of the corresponding curve for the lysis of red cells, the relation between the percentage hemolysis and the amount of lysin added is a sigmoid one (Ponder, 1934a)   It is impossible to decide, however, whether the curve in Fig 3 is sigmoid or not, since the points at the upper end of the curve are subject to very considerable experimental error, and since any sigmoid relation which might be found would almost certainly be an extremely asymmetrical one [2]

2 *The Bile Salts* —Sodium taurocholate and sodium glycocholate,

[2] The respiration of the cells before the addition of the lysin or the '100 per cent respiration, can be relied upon to be maintained for at least 3 hours with no greater variation than ±5 per cent   The respiration for any 10 minute period after the addition of the lysin can be measured with about the same accuracy   Thus a point towards the upper end of the curve might be affected with a ±10 per cent error

in suitable concentrations, produce effects very similar to those of saponin   Table III shows the relation between the quantity of bile salt added to the systems and the amount of residual respiration

If plotted, the figures in Table III give curves similar to that in Fig 3 for saponin   So far as their effect on white cell respiration is concerned, the two bile salts are apparently about equally effective, and about one-eighth as effective as saponin   In the case of hemolysis of red cells, sodium taurocholate is usually a little more active than is the glycocholate, and saponin about ten times as effective as the bile salts (Ponder, 1934*a*)   The order of effectiveness in reducing white cell O_2 consumption is accordingly much the same as that for producing hemolysis

A further point about the action of the bile salts as compared with that of saponin ought to be remarked upon   Instead of the residual respiration falling off roughly exponentially with time after the addition of the lysin, a new level of respiration is attained very quickly after the addition of the bile salts (within 10 minutes), and tends to be steadily maintained   This suggests that the course of reaction between saponin and the white cells is different from that between the bile salts and the white cells, the former reaction being steady and continuous, while the latter is more immediate   A similar difference is met with in the case of the "absorption" of these lysins at red cell interfaces, the bile salts becoming rapidly concentrated at the surfaces, while saponin is not "initially absorbed" at all (Ponder, 1934*b*)   There is difficulty in investigating the point fully because of the instability of the bile salts as lysins

3 Sodium Oleate —This very unstable lysin is effective in reducing white cell O_2 consumption in a dilution of 1 in 100 or less, but we have not obtained quantitative relations

4 Digitonin —This hemolysin produces hemolysis only in solutions which are nearly saturated, and, white cell respiration being affected only by concentrations much greater than are necessary to cause hemolysis, it is not surprising that even a saturated solution of digitonin (about 1 in 100,000) produces no observable effect on respiration

5 Amboceptor and Complement —The addition of guinea pig complement to white cells sensitized with anti-rabbit amboceptor pro-

duces no diminution in the O_2 consumption of the system   In these experiments the cells received 50 hemolytic doses of amboceptor, and the complement-containing serum added was undiluted

   6 Freezing and Thawing —Freezing and thawing the white cell suspension three times results in the O_2 consumption being reduced

TABLE IV

NaCl	Respiration
per cent	*per cent*
0 95	100
0 85	85
0 75	78
0 60	60
0 45	45
0 15	10
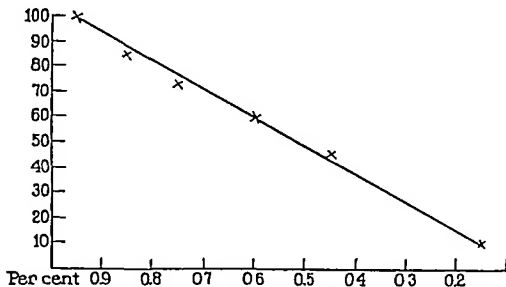
FIG 4 Ordinate respiration as a percentage of that in 0 95 per cent NaCl, abscissa NaCl concentration per cent

to about 5 per cent of its initial value   This small residual respiration may be due to the presence of cell or nuclear fragments

   7 Hypotonic Saline —Placing white cells in hypotonic solutions reduces their O_2 consumption, and Table IV shows the O_2 consumption, expressed as a percentage of that in 0 95 per cent NaCl, in systems of various tonicity

   The points lie very nearly on a straight line (Fig 4)   The curve

bears no resemblance to the sigmoid relation between percentage hemolysis and tonicity, which is scarcely surprising, for white cell metabolism is not an all-or-none property like the maintenance of the integrity of the red cell membrane   The possibility that the reduction in O_2 consumption in hypotonic media is due to some of the less resistant cells being cytolyzed while others are unaffected can easily be disposed of on the grounds of microscopical evidence   The white cells in a system in 0 5 per cent NaCl, in which there is about 50 per cent reduction in the O_2 consumption, are all greatly swollen and obviously partially cytolyzed, granules in the cytoplasm show Brownian movement, and the nuclei are very prominent [3]

The Quantity of Lysin Combined with the Cells

The remainder of this investigation is concerned with relating the effect of a lysin on white cell O_2 consumption with the quantity of lysin actually combined with the cells   It will be clear that the total quantity of lysin introduced into the system is not necessarily a measure of the quantity which reacts with the leucocytes, for the lysin may be concentrated at the cell surfaces, just as it often is in the case of red cell suspensions (Ponder, 1934b)   An investigation of the extent of the concentration, however, is possible with saponin only, for the other lysins referred to in this paper are too unstable to be used in quantitative work

Methods

These are substantially the same as methods which have been described already (Ponder, 1934b), and the principle underlying them is as follows   A known concentration of saponin is added to the white cell suspension, and the cells and lysin are allowed to react at constant temperature (37°C) for a known time, the cells are then thrown down, the supernatant fluid removed, and the concentration of saponin in it found by adding red cells and observing the time required for their complete hemolysis   The difference between the amount of lysin introduced into

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[3] After the addition of saponin, the bile salts, and the soaps, the cells show obvious cytolytic changes   The cytoplasm shows no structure, and one gets the impression that it has disappeared, leaving a very prominent nucleus inside an apparently empty space bounded by the cell envelope   There does not seem to be any diminution in the number of the cells   Suspensions of these injured cells show very marked agglutination, both microscopically and macroscopically

the system and that found in the supernatant fluid gives the quantity which has been used up in the time allowed for the reaction, principally by "combining with" the cells

In practice, the first step is to plot a time-dilution curve at 37 C showing the time required for complete lysis of 0 4 cc of a "standard red cell suspension[4] by 1 6 cc of various dilutions of saponin starting with a dilution of about 1 in 1000 One dilution of saponin is then selected, and 1 cc of it is added to each of a number of tubes containing 2 cc of the white cell suspension The lysin and cells are allowed to react for various times (1 minute 5 minutes, 30 minutes, etc), and at the end of the time the tube is spun gently so as to throw down the cells 1 6 cc of the supernatant fluid is then removed 0 4 cc of the standard red cell suspension added and the time for complete lysis determined Reference to the time-dilution curve gives the quantity of saponin present in the supernatant fluid In this way we can find out how the lysin disappears from the fluid surrounding the cells with the passage of time, and by repeating the experiment with different dilutions of saponin, we can construct curves showing the course of the disappearance for many different initial quantities of lysin

Unfortunately this procedure is complicated by the fact that the supernatant fluid obtained from a white cell suspension contains substances (probably of a protein nature) which react with saponin, and so the amount of saponin Δ_1 which disappears when the lysin is added to a white cell suspension is made up of two quantities an amount Δ_2 which has combined with the cells and an amount Δ_3 which has combined with the inhibitory substances in the fluid surrounding the cells The latter amount can be found by adding 1 cc of a known dilution of saponin to 2 cc of supernatant fluid obtained from the white cell suspension and then finding the amount of lysin rendered inert by taking 1 6 cc. of the mixture adding 0 4 cc. of the standard red cell suspension, finding the time for complete hemolysis and referring to the time-dilution curve for the amount of lysin present Subtraction from the amount originally introduced gives Δ_3 and subtraction of Δ_3 from Δ_1 gives Δ_2 the amount of lysin combined with the cells under the particular conditions of the experiment

RESULTS

Fig 5 shows the course of the disappearance of saponin for three lysin dilutions reacting with the cells of a suspension containing 68,000 white cells per mm³ The range of lysin dilutions over which such curves can be obtained with any pretense to accuracy is unfortunately very small, for if the initial amount of lysin is too great there may be

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[4] This is a suspension prepared by suspending the thrice washed cells of 1 cc of rabbit blood in 20 cc. of 1 per cent NaCl For further details of technique see Ponder, 1934a

so much left after absorption that it is impossible to determine it accurately because the added red cells hemolyze so rapidly, while if the amount is too small so much may be absorbed that the remainder is insufficient to hemolyze the added red cells at all

The curves show a striking resemblance to those shown in Fig 1 of a previous paper (Ponder, 1935) dealing with the disappearance of
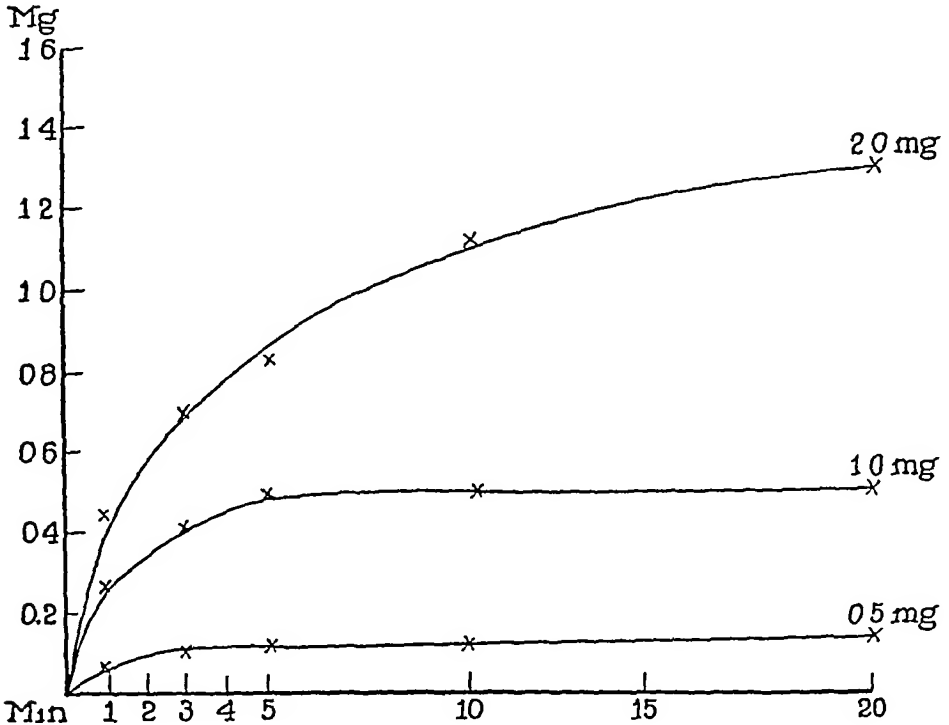
FIG 5 Ordinate, lysin disappearing from the system, in milligrams, abscissa, time in minutes The figures opposite the curves show the amount of saponin initially added

saponin during stromatolysis and the absorption of the lysin at the surfaces of ghosts The disappearance of lysin is rapid at first, and tends to reach a final value asymptotically, the particular amount which finally disappears being a function of the amount initially present, the time, moreover, which it takes for the final value to be reached increases as the initial concentration increases An important point is that the disappearance apparently ceases while there is yet a large amount of saponin free in the system, and this, together with all

the other properties of the reaction, indicates that the combination of saponin with white cells is essentially similar to its combination with red cell stromata, and of the nature of a "pseudo adsorption" or "anomalous" adsorption (see Ponder, 1935)

It will be observed that, in the case of these quantities of saponin at least, the disappearance of lysin is virtually complete after 30

TABLE V

Saponin added	Δ_1	Δ_2	Δ_3	Residual respiration
mg				*per cent*
10 0	4 100	3 800	(0 300)	0
2 0	1 767	1 434	0 333	9
1 0	0 884	0 509	0 375	32
0 5	0 448	0 148	0 300	64
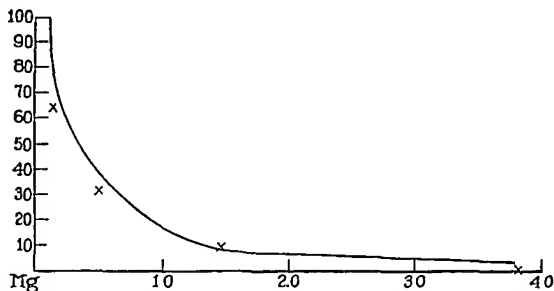
FIG 6 Ordinate, residual respiration 30 minutes after addition of saponin, abscissa lysin combined with the cells after 30 minutes The curve is the hyperbola $R.\Delta = 12\ 0$

minutes at 37°C At the end of the same time, there is also a considerable reduction in the O_2 consumption of the systems, and so, as a final step, we can relate the O_2 consumption of the cells to the quantity of saponin combined with them This is done by carrying out, on the same white cell suspension, an experiment divided into two parts the first part consists in finding the effect of the lysin on the

O_2 consumption 30 minutes after its addition, and the second consists in finding the quantity of lysin absorbed after 30 minutes under the same conditions as regards temperature, concentration, etc   Table V shows the results of such an experiment

The figures in the columns for Δ_3 show that the quantity of saponin rendered inert by the substances in the fluid surrounding the cells does not depend on the initial lysin concentration (the value in brackets could not be found experimentally), and these values, when subtracted from Δ_1, the total amount of saponin which has disappeared, give Δ_2, the quantity of saponin combined with the cells   This value, in milligrams, can then be plotted against the residual respiration R to give Fig 6   Although there are so few points, there is no doubt as to the general shape of the curve, which is so close to that of the rectangular hyperbola

$$R \, \Delta_2 = \text{constant} \tag{1}$$

that it would be almost impossible to discover a difference experimentally   Without unduly stressing the point, it may be remarked that such a relation would be expected if the saponin were adsorbed at surfaces which were the seat of oxidative processes

SUMMARY

Substances such as saponin, the bile salts, etc , which produce lysis of red cells also produce cytolysis of white cells from rabbit peritoneal exudates, the arbitrary criterion of their cytolytic effect being their ability to depress the O_2 consumption of the leucocytes   The amount of cytolysis increases regularly as the amount of the added lysin is increased, and sufficiently large quantities of saponin, sodium taurocholate, sodium glycocholate, or sodium oleate are capable of virtually abolishing the O_2 consumption altogether   At the same time, it can be shown that a lysin such as saponin is used up in combining with the white cells in much the same way as it is used up in combining with red cells, and the reduction in oxygen consumption appears to be roughly proportional to the amount so combined   The action of these lytic substances on white cells, in fact, is very similar to their action on red cells, due allowance being made for the fact that the

cytolysis of the white cell is probably not an all-or none process like hemolysis

White cell respiration is also depressed in hypotonic solutions, the respiration being virtually linear with the tonicity

We have to thank Dr L Reiner for preparing the anti rabbit amboceptor for us

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GROWTH SUBSTANCE CURVATURES OF AVENA
IN LIGHT AND DARK

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I

INTRODUCTION

Since it had been shown (Van Overbeek, 1932, 1933) that growth substance curvatures of *Raphanus* hypocotyls offered an easy way to study light growth responses, the question arose whether this was true for *Avena* coleoptiles too   By growth substance curvatures are meant such curvatures as appear when growth hormone is applied unilaterally to a coleoptile, hypocotyl, etc   If the growth substance curvatures of *Raphanus* hypocotyls which have occurred in the dark are compared with those that have occurred in the light (exposed from all sides), the former (dark) are always the larger   The ratio of the curvatures in dark and light depends on the amount of light, on the concentration of the hormone applied, etc   Furthermore it has been shown clearly that the difference in growth in light and dark has an important part in the explanation of the phototropic curvature of *Raphanus*

In *Avena*, on the other hand, the light growth responses seem to have a minor part, if any, in the explanation of the phototropic curvature   Went (1928) was able to show that an unequal distribution of the growth substance, caused by unilateral exposure to light, is sufficient to explain the entire phototropic curvature of the *Avena* coleoptile   This unequal distribution occurs in the tip, which is solid, and, therefore, more fit for lateral transport of the growth substance than the hollow lower parts of the coleoptile   Cholodny (1933) was able to show that it was possible to obtain a phototropic curvature in

Avena under circumstances which did not cause a light growth response

The growth substance curvatures of *Avena* coleoptiles in light and dark may not be important for the problem of phototropism, but it certainly is worth while to study them in order to increase our knowledge of the physiology of the coleoptiles

On the basis of the present investigations, moreover, it has become possible to answer practical questions which had never before been answered satisfactorily   (1) Do decapitated coleoptiles such as those used in the growth substance test react in the same way to a given amount of growth substance after they have been photographed, *i e*, exposed to a small amount of light?   The answer is no, their sensitivity is higher because the formation of new growth substance in the decapitated coleoptiles is inhibited by light   This "regeneration" of the growth substance formation decreases the sensitivity of the coleoptiles to growth hormone   Skoog (1937), however, has developed a method to obtain "regeneration free" plants   These plants can be exposed hourly to a small amount of light without the slightest difference in sensitivity to growth substance

(2) Another question is answered by the present investigations on the growth substance curvatures in dark and light, *viz* can hetero-auxin be used without limit as a substitute for auxin-*a* and auxin-*b*? Hetero-auxin is a substance produced by fungi as shown by Kogl and Kostermans (1934) and by Thimann (1935*a*)   Auxin-*a* and auxin-*b* are present in higher plants (Kogl, Haagen-Smit, and Erxleben (1934))   In a recent study by Haagen-Smit and Went (1935) and by Thimann (1935*a*) it has been shown that in addition to hetero-auxin a large number of other substances which are *not the natural growth substance of Avena* and pea plants do stimulate the growth apparently in the same way as auxin-*a*, the only difference being that these other substances require a higher concentration to act in the same way that auxin-*a* does   Furthermore it has been shown by Skoog and Thimann (1934) that both hetero-auxin and auxin-*b* are able to inhibit the development of lateral buds   Went (1934) and Thimann and Went (1934) have shown that the root formation of pea cuttings can be increased greatly by application of hetero-auxin as well as of auxin-*a* Cooper (1935) obtained a stimulation of root formation in lemon and

other woody cuttings after application of hetero auxin   All these
facts seemed to indicate that hetero auxin can act as a substitute for
auxin *a* in every sense   Below, however, it will be shown that if in
Avena coleoptiles auxin *a* is replaced by hetero auxin, growth is
not inhibited after exposure to light   It will be shown, furthermore,
that there are considerable differences in the destruction of both
hormones

The present investigations were started at the Rijks Universiteit in Utrecht,
Holland in the early part of 1934   They were continued, after an interval of
about a year, at the California Institute of Technology in Pasadena

<center>II</center>

A   Experiments Carried out in Utrecht

Method —A detailed description of the experimental set up is given in an earlier
paper (Van Overbeek 1933[1])   Briefly the circumstances under which the experi
ments were carried out were as follows   Temperature 21 C and humidity about
90 per cent   The experiments in the dark were carried out in a dark room with
controlled conditions   The room was lighted with orange yellow light of wave
lengths larger than 546 mμ The experiments in the light were done in an incu
bator which was kept at the same constant temperature and humidity as the dark
room   In order to expose the plants uniformly on all sides, they were placed on
a disk revolving in a horizontal plane   The light source consisted of two incan
descent lamps of 500 watts each which were placed at 80 cm distance from the
objects   The plants were shielded from the heat of the lamps by a set of glass
plates and water containers   Under these conditions one lamp radiates an
amount of energy of 200 erg/cm per second upon the plants

The *Avena* plants used were   Victory oats from Svalof   They were grown
under standard conditions (see for example Went 1935a)

The auxin used was pure auxin-*a* supplied by Prof F Kogl and Dr A J
Haagen Smit

1 Continuous Exposure Starting at the Second Decapitation —When
the coleoptiles were about 3 cm long they were prepared in the follow
ing way   The tip of the coleoptiles was cut off (first decapitation) and
1½ hours later a fresh cut surface was made (second decapitation) and
the primary leaf was pulled loose   Immediately after this an agar
block containing auxin *a* was applied on one side of the cut surface of
each coleoptile   After this preparation one set of plants was exposed

[1] P 550

on all sides to the light, and another set was kept in the dark room The curved plants were photographed 110 minutes after the blocks had been put on and the experiment was stopped   Later the curvatures of these plants were measured [2]

Fig 1 shows the results of three experiments carried out as described above   In these experiments the concentration of the auxin-a is plotted on the abscissa   It is clear that *the growth substance curvature of the coleoptiles which were exposed on all sides to light is smaller than those of the non-exposed plants*
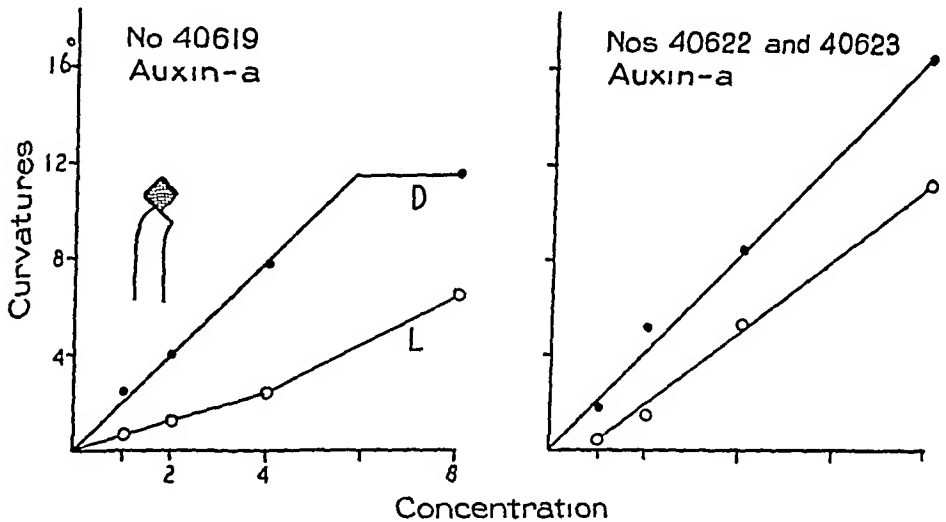
FIG 1 Auxin-a curvatures of twice decapitated *Avena* coleoptiles in dark (black dots) and exposed on all sides to light (circles)   Abscissa relative concentration of the hormone   Each point is the average of the curvatures of twelve plants [3]

2 Continuous Exposure Starting at the First Decapitation —In order to see what will happen if the plants are exposed immediately after the first decapitation until the end of the experiment, the following experiment was made   Plants were decapitated and immediately after this decapitation one set of plants was exposed to the light

[2] To secure subjective measurements the photographic records were measured out "blindly" (without knowing to what the record referred)

[3] All figures in the graphs and tables of this publication represent average values of twelve plants, unless the contrary is stated

(without growth substance being applied) 1½ hours later the plants were decapitated again and auxin *a* blocks were applied Photographs were made 110 minutes after this and the experiment finished The results, shown in Table I, show that the exposed plants have on an average the same curvature as the non exposed controls Table I also shows the results of experiments similar to those described in the section entitled Continuous exposure starting at the second decapitation

To study the different behavior of *Raphanus* as compared to *Avena*, the same experiments which were carried out and described in this section for *Avena* were repeated with *Raphanus* The preparation of

TABLE I

Auxin A Curvatures of Twice Decapitated Avena Coleoptiles

Auxin application after second decapitation Three sets of plants (1) Exposed continuously from the first decapitation, (2) exposed from the second decapitation, and (3) control in dark. Temperature 21°C 2 lamps of 500 watts at 80 cm Photographed 110 minutes after the growth substance was applied

No	(1) Exposed from first decapitation until end of experiment	(2) Exposed from second decapitation until end of experiment	(3) Dark
40 416	10 5	6 4	10 5
	—	5 8	11 8
	—	—	13 0
40 614	11 6		9 4
	14 2		13 9
40 625	4 3		3 6
	4 8		4 0
Average	9 7	6 1	10 2

the *Raphanus* hypocotyls was the same as has been described in the earlier paper Table II shows the results Whether the hypocotyls were exposed 2 hours before and 2 hours during the growth substance action, or the plants were exposed during the action of the hormone only, did not have any influence upon the growth substance curvature In both cases the average value of the growth substance curvatures in the dark was 12, and 5 in the exposed ones The same value was found during the experiments described in Fig 27 in the earlier paper

3 Plants Exposed between the First and Second Decapitation Only — After the first decapitation one set of plants was exposed   1½ hours later the plants were brought back into the dark room and decapitated again, where upon auxin-*a* was applied on one side of the cut surface

TABLE II

Auxin-A Curvatures of Raphanus Hypocotyls

Auxin applied 2 hrs before the end of the experiments   Exposed 4, 2, and 0 hrs before the end of the experiment   Temperature 21°C   2 lamps of 500 watts at 80 cm   Photographed 2 hrs after application of auxin   Nos 40,606, 40,611, and 40,613

4 hrs exposed (average of 9 × 12 plants)	4 9 ± 0 26
2 hrs exposed (average of 7 × 12 plants)	5 2 ± 0 60
Not exposed (average of 8 × 12 plants)	12 1 ± 0 44

TABLE III

Auxin-A Curvatures of Avena Coleoptiles

Auxin applied after second decapitation   One set of plants exposed between the first and second decapitation (before growth substance application), other set is dark controls   Temperature 21°C   2 lamps of 500 watts at 80 cm   Photographed 110 min after growth substance application   No 40,423   Average of 6 × 12 plants for each set

Exposed (between first and second decapitation only)	8 9 ± 0 33
Not exposed	6 8 ± 0 31

TABLE IV

Auxin-A Curvatures of Raphanus Hypocotyls

2 hrs before the growth substance was applied one set of plants had been exposed for 2 hrs , another set was kept in the dark room all the time   Growth substance application 2 hrs before the end of the experiment   Temperature 21°C   2 lamps of 500 watts at 80 cm   No 40,612   Average of 4 × 12 plants for each set

Exposed (2 hrs before growth substance application only)	12 0 ± 0 42
Non-exposed	12 5 ± 0 63

of the coleoptiles   The controls, which had not been exposed, were treated in the same way   The curvatures were photographed 110 minutes after the blocks had been put on   The results are shown in

Table III   The plants that had been pre exposed show a larger cur
vature than the non exposed controls

In one further respect *Raphanus* also reacts differently from *Avena*
coleoptiles   It does not show an effect of exposure to light before the
auxin *a* had been applied as Table IV shows   *Raphanus* obviously
reacts only (with a growth inhibition) if the growth substance acts
during the exposure

B Experiments Carried out in Pasadena

4 Method—We have tried to carry out these experiments under conditions
resembling those in Utrecht as closely as possible   The experiments in light were
carried out in a room with controlled conditions   The lamps were placed in the
room itself and were cooled by running water   Two to four lamps of 200 watts
each were used at a distance of 125 cm. from the objects   The oats were Victory
oats from Svalof   Two important differences, however, had to be made   (1)
Due to the instability of auxin-*a* which is made only at Prof Kogl s laboratories
in Holland, the active pure substance could not be obtained here   Therefore
pure synthetic hetero auxin was used which was supplied by Drs K V Thimann
and J Koepfli   (2) Due to the hot summers in Pasadena it was impossible to
maintain a constant temperature below 27 C in the experimental rooms, which
are not equipped with a cooling system

*5 Continuous Exposure Starting at the Second Decapitation, No
Difference between the Hetero Auxin Curvatures in Light and Dark*—If
the experiments of the section entitled Continuous exposure starting
at the second decapitation were repeated, but at 27°C and with
hetero auxin instead of auxin *a*, no difference between the curvatures
in light and dark could be found   In order to find which one of the
two factors was responsible for the non-occurrence of the smaller
curvature in the light, the temperature of the rooms was lowered
By bringing ice into the rooms and stirring the air by means of electric
fans a fairly constant temperature around 23°C could be main
tained   The experiment was then repeated   The *Avena* coleoptiles
were decapitated, and after 1½ hours again decapitated   Agar blocks
containing hetero auxin were put on one side of the cut surface of
the coleoptile stumps   One set of the plants treated in this way was
exposed on all sides and a control set was kept in the dark room   The
experiments were stopped 110 minutes later and the plants photo
graphed   Fig 2 shows the results   The curvature in the light is

still not smaller than in the dark   Hence the kind of growth hormone used must be an important factor in these experiments

6 *Continuous Exposure Starting at the Second Decapitation, the Auxin-A and B Curvature is Smaller in the Light Than in the Dark* —As no pure auxin-*a* was available a less refined product had to be used In order to obtain such a product a method was followed similar to that used by Kornmann (1935) who extracted corn starch with water Coarse corn meal was extracted with distilled water for about 12 hours at 3°C   Plain agar blocks were soaked in the extract for a few hours
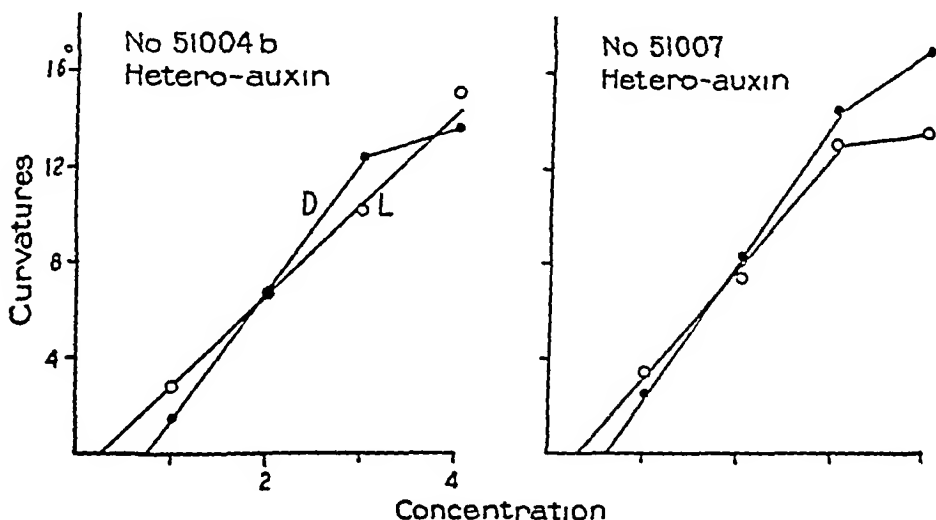
FIG 2 Hetero-auxin curvatures of twice decapitated *Avena* coleoptiles in dark and exposed on all sides to light

If these agar blocks were diluted twice, blocks of 1 x 2 x 2 mm gave a curvature of about 10° in the standard test   From the experiments of Kogl, Erxleben, and Haagen-Smit (1934) it is known that corn oil contains a large quantity of auxin-*a* and *b*   It is very probable therefore that the growth hormone which we extracted from corn meal consists of a mixture of the auxins-*a* and *b*   The growth hormone extracted from corn meal will be called in this paper auxin-*a* and *b* and the curvature caused by this hormone an auxin-*a* and *b* curvature

If now the experiments described in the last section were repeated again with this auxin-*a* and *b* as growth hormone, results similar to those obtained with pure auxin-*a* (Section 1) were obtained   Fig 3

shows two of these experiments, the curvature in light is smaller than in the dark Figs 4a and 4b show similar results with deseeded (regeneration free) plants In the experiment of Fig 4a auxin a and
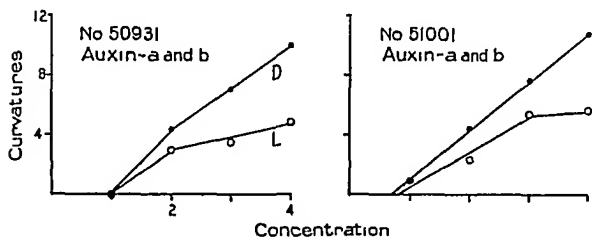
FIG 3 Auxin-a and b curvatures of twice decapitated *Avena* coleoptiles in dark and exposed on all sides to light
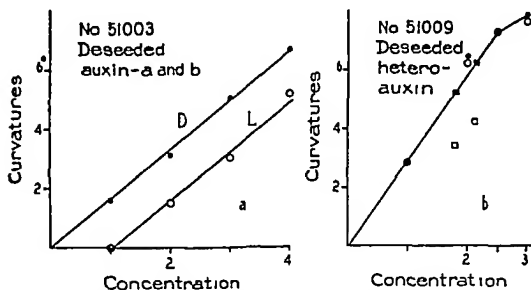
FIG 4 Growth substance curvatures of twice decapitated and deseeded *Avena* coleoptiles (a) Auxin-a and b curvatures (b) Hetero auxin curvatures, but with auxin a controls The black dots indicate the hetero-auxin curvatures in the dark The open circles indicate the hetero auxin curvatures in the light The black squares indicate the auxin a curvatures in the dark and the open squares indicate the auxin a curvatures in the light

b was used, while in the one of Fig 4b hetero auxin was used In the latter experiment, however, a control with auxin a and b was made at the same time The black squares show the curvatures with corn meal auxin in the dark, the open squares show the same in the light

7 Continuous Exposure Starting at the First Decapitation, and Exposure between the First and Second Decapitation only, in Both Cases the Hetero-Auxin Curvature is Larger in the Light Than in the Dark —In Section 2 it has been shown that if *Avena* coleoptiles are exposed continuously from the first decapitation and the auxin-*a* blocks are put on after the second decapitation, the curvatures obtained are the same in the light as in the dark   If the experiment is repeated, however, with hetero-auxin instead of auxin-*a*, the curvature is larger in the light than in the dark as is shown in Table V   In this table the results of experiments similar to those described in Section 3 are also given   The experiments described there were with plants which were exposed on all sides between the first and second

TABLE V

Hetero-Auxin Curvatures of Twice Decapitated Avena Coleoptiles

Three sets of plants, (1) exposed continuously from first decapitation, (2) exposed between first and second decapitation only, (3) controls in dark   Temperature 23°C   4 lamps of 200 watts at 125 cm   Photographed 1½ hrs after growth substance application   Nos 51,125, 51,126   Average of 7 × 12 plants for each set

1 (Exposed from first and after second decapitation)	9 6 ± 0 81
2 (Exposed between first and second decapitation only)	8 9 ± 0 61
3 (Dark)	6 6 ± 0 30

decapitation (1½ hours) only   After the second decapitation auxin-*a* blocks were put on the cut surface of the coleoptile stumps and 110 minutes later the pre-exposed plants had a larger curvature than the non-exposed controls   Table V shows that this experiment can be repeated with hetero-auxin with the same result

8 Growth Substance Curvatures of Once Decapitated Avena Coleoptiles, Explanation —Van der Wey (1931) in a detailed investigation on the influence of decapitation upon the growth substance curvature, showed that the growth substance curvature obtained with the same amount of growth hormone is larger in plants that had been decapitated two or three times than in plants which had been decapitated only once, especially if the agar blocks containing the hormone are applied right after the first decapitation   If the tip of a coleoptile is

cut off the stump still contains a large amount of hormone which dis
appears gradually   1½ hours after the tip has been cut off almost all
the hormone in the stump has disappeared, but at the same time the
stump is starting to produce growth hormone again   This regenera-
tion, however, can be suppressed by the second decapitation   There
fore if the cells of the stumps are empty as far as growth substance
is concerned a larger growth substance curvature occurs than if the
cells have a high growth substance content though in both cases the
same amount of growth substance is used

Let us analyze now the results obtained from the following experi
ment   *Avena* plants are divided into four groups   The coleoptiles

TABLE VI

Hetero Auxin Curvatures of Once and Twice Decapitated Avena Coleoptiles

Temperature 27 C   4 lamps of 200 watts at 125 cm   Photographed 110 min
after growth substance application

No	Once decapitated		Twice decapitated	
	Exposed	Dark	Exposed	Dark
50 902	6 7	3 0	5 8	6 6
	—	3 8	5 3	6 0
	—	3 0	5 3	—
50 909a	7 7	2 8	7 6	6 0
	5 0	2 2	6 7	7 8
Average	6 5	3 0	6 1	6 6

of two of them are decapitated, the remaining groups are left intact
After 1½ hours the first two groups are decapitated again and agar
blocks containing hetero auxin are applied on one side of the cut
surfaces   One of these groups is placed in the light, the other one
remains in the dark room   The two other groups are now decapitated
also, but for the first time, and hetero auxin of the same concentration
and in the same way is applied to these groups right after the first
decapitation   One of these groups is placed in the light too, whereas
the other one stays in the dark room   The plants were photographed
110 minutes after the blocks were put on   Table VI shows the sur
prising result that the curvatures of twice decapitated plants and
the just once decapitated but exposed plants are the same   The

curvatures of the just once decapitated plants in the dark have a smaller curvature than the twice decapitated plants in the dark As has been shown above, this is due to the fact that during the period between the first and second decapitation the growth hormone left behind in the plant after the tip is cut off, has disappeared It is reasonable to suppose that the growth hormone left behind in the plant after first decapitation, has disappeared also in the plants that have been exposed to light This growth hormone is very probably auxin-*a* or (and) its close relatives, because Kogl, Haagen-Smit, and Erxleben have shown that the hormone present in *Avena* coleoptiles has the same molecular weight and other properties as auxin-*a* Another interesting fact which can be concluded also from Table VI, is that hetero-auxin apparently is much less affected by light than

TABLE VII

Auxin-A and B Curvatures of Once Decapitated Plants

Temperature 23°C 4 lamps of 200 watts at 125 cm Photographed 1½ hrs after growth substance application No 51,127 Average of 3 × 12 plants for each set of plants

Exposed	8 6 ± 0 64
Dark	10 3 ± 0 71

auxin-*a* If this point of view is right, once decapitated plants to which auxin-*a* and *b* is applied must show a smaller $\frac{\text{Light}}{\text{Dark}}$ ratio than the once decapitated plants to which hetero-auxin has been applied (Table VI) Table VII gives the answer The curvature of the plants which have been exposed to light and to which auxin-*a* and *b* has been applied is even smaller than that of the non-exposed controls Apparently also a part of the auxin-*a* and *b* which was added to the plants has disappeared before it could accelerate the growth of the cells of the coleoptile stump, therefore the auxin-*a* and *b* probably is inactivated (destroyed) in the light Another support for the idea that the auxin-*a* is destroyed in the light, is the fact that it is much more easily destroyed by enzymes of the plant than hetero-auxin, as

will be shown below   The results of the preceding sections are summarized in the following scheme

Once Decapitated	*Twice Decapitated*

Twice Decapitated

(1) *Dark*
    (a) Hetero auxin curvature 6 6
        (Tables V and VI)
    (b) Auxin curvature, *6 6*
        (Tables I and III)

Dark (2)
(a) Hetero auxin curvature 3 0
    (Table VI)
(b) Auxin curvature *3 0*
    (Table VII)

(3) *Light*
    (a) Hetero auxin curvature 6 1
        (Table VI)
    (b) Auxin curvature 3 9
        (Table I)

(4) *Dark*
    (a) Hetero auxin curvature 8 9
        (Table V)
    (b) Auxin curvature *8 6*
        (Table III)

Light (5)
(a) Hetero auxin curvature 6 6
    (Table VI)
(b) Auxin curvature 2 5
    (Table VII)

(6) *Light*
    (a) Hetero auxin curvature 9 6
        (Table V)
    (b) Auxin curvature 6 3
        (Table I)

On the left hand side of the scheme is indicated whether the plants between the first and second decapitation were in the light or in the dark   On the right hand side is indicated whether after the second decapitation the plants were in the light or in the dark   There are two groups of dark plants (1) and (4), each of which has a different previous history   The group (4) has been exposed before the second decapitation, the group (1) has not   The same holds for the "light groups" (3) and (6)   In each group the hetero auxin curvature (a) and auxin a or auxin a and b curvatures (b) are indicated   Each figure in this scheme is directly comparable with any other figure in the scheme   The values for the hetero auxin curvatures are obtained directly from the tables as indicated in each group, the (1) group serv

ing as a "standard" The auxin-*a* curvatures, however, are reduced to this standard (1*a*)

In Table I for example for the twice decapitated plants which were not exposed, a value 10 2 is found This value is reduced to 6 6 in order to make the auxin-*a* curvatures directly comparable to the hetero-auxin curvatures The value 6 1 of Table I must be multiplied with the factor $\dfrac{6\,6}{10\,2}$ in order to be comparable with the standard values of group (1) and, therefore, 3 9 is obtained for (3*b*) The same is done for the 9 7 value of Table I which gives 6 3 in group (6) under (*b*) In Table III we find a dark value of 6 8 which has to be reduced to 6 6 Therefore the value in group (4*b*) will be $\dfrac{6\,6}{6\,8}$ 8 9 = 8 6 At last the value 10 3 (Table VII) was reduced to 3 0 of group (2*a*) and, therefore, the value 8 6 of Table VII is reduced to $\dfrac{3}{10\,3}$ 8 6 = 2 5 of group (5*b*)

In the beginning of this section the conclusion was drawn that auxin-*a* and closely related substances (which are stable to light by themselves) are destroyed within the plant on exposure to light The hetero-auxin is not destroyed, or more probably is much less destroyed than the auxin-*a* These conclusions were drawn from experiments on once decapitated plants

The experimental data summarized in the scheme may now be considered in regard to these conclusions If the groups (1) and (3) are compared, we find the hetero-auxin curvature in the light slightly lower than that in the dark This may indicate that indeed a small amount of hetero-auxin is destroyed in the exposed plant The auxin-*a* curvature of group (3), however, is much smaller than that of group (1) This is caused according to the conclusions mentioned above by the destruction of auxin-*a* in the exposed plants.

If group (4) is compared with group (1) a higher curvature in group (4) is obvious Both the hetero-auxin and auxin-*a* curvatures are higher in the plants which had been exposed previously Because, during the growth substance action the plants were in the dark, no auxin-*a* was destroyed and therefore the value (4*a*) is practically the same as the value of (4*b*) The results show another aspect, however, if the plants are exposed to light during the action of the growth substance This is manifest if the groups (1) and (4) are compared with

group (6)   The values of $(4a)$ and $(6a)$ are practically the same (mean error rather high, Table V)   The auxin a curvature of group (6), however, is considerably lower than in group (4)   This is easy to understand if it is assumed that the auxin a is destroyed in plants which are exposed to light   Why the response to growth hormone is higher in the groups (4) and (6) than in the group (1) can also be explained in terms of destruction of growth substance by light   The plants of groups (4) and (6) have been pre exposed and hence have a smaller growth hormone content than group (1) which has been kept in the dark all the time   Consequently the plants of groups (4) and (6) are more sensitive to growth substance than the ones of group (1) as is explained at the beginning of this section

The following general conclusions can be drawn from these considerations   (1) The response of the plant to growth hormone (both types of auxins) can be shown by the hetero auxin curvature   (2) Superimposed upon this response is the destruction of auxin-a in exposed plants

If the hetero auxin curvature in the dark is Hd, the one in the light Hl, and the auxin a curvature in the dark is Ad, then according to these general conclusions the auxin a curvature of exposed plants can be expressed $\dfrac{Hl}{Hd}$ $(Ad - Dl)$ In which Dl is the destruction of the auxin a in the exposed plant

9 The Destruction of Hetero Auxin and Auxin A and B —In an earlier paper dealing with dwarfs of corn (Van Overbeek (1935)) a method was developed to determine the destruction of growth substance by sections of plants in agar blocks   Sections of coleoptiles, mesocotyls, etc were placed with their basal cut surface on agar blocks containing growth hormone   If after a certain time the sections were removed and the blocks analyzed, some of the hormone had disappeared from the blocks   Since then Kornmann (1935) has published similar results   It is certain that enzymes set free from the cells at the cut surface have at least a part in the destruction of the hormone, because rinsing of the cut surface with water reduces the destruction   The following evidence indicates that this inactivation of the growth hormone is due to an oxidative process   Sections (about 5 mm long) were cut from the apical part

and from the basal part of a mesocotyl of corn   These sections were placed with their basal cut surface on wet filter paper for $1\frac{1}{2}$ hours in order to set free the growth hormone they might contain   These sections were then placed on blocks containing hetero-auxin for 1 hour   After the sections were removed, the blocks were analyzed and it could be shown that more hormone was destroyed in the blocks with which the basal sections had been in contact than in the blocks on which apical sections had been put (Table VIII)   If with similar sections a peroxidase test was made, the peroxidase activity was proved to be higher in the basal sections than in the apical sections   This peroxidase test was carried out as follows   Agar blocks were soaked in a benzidine solution to which a small amount of H_2O_2 had been added   If peroxidase is present in the cut surface of the sections the benzidine in the block will be oxidized when the sections are

TABLE VIII

Inactivation of Hetero-Auxin by Apical and Basal Mesocotyl Sections

No of experiment	41,201	41,130	41,204
Concentration of hormone started with	9 0	18 0	16 0
Left over in blocks with,			
Mesocotyl tips	7 2	11 3	15 0
Mesocotyl bases	1 5	3 8	6 0

brought into contact with the blocks   Since the oxidized benzidine is colored, the darker the color in the block is, the higher the peroxidase activity on the cut surface of the section was   Fig 5 shows a photograph of such a peroxidase test   The picture is a negative and therefore the whiter the spots (places where the sections made contact with the agar block) are, the higher the peroxidase activity was

If the destruction of auxin-*a* and hetero-auxin are compared by the method mentioned in the beginning of this section, a higher percentage of the auxin-*a* and *b* than of the hetero-auxin is destroyed   Table IX shows the results of these experiments for *Avena* coleoptiles   In Experiment 51,029*a* 5 day old plants were used   The coleoptile was cut off and decapitated and placed with its basal cut surface in water for $1\frac{1}{2}$ hours   Then from the apical part of the coleoptiles two sections 5 mm long were cut, which were placed with their basal

cut surface on blocks containing hormone   After 2 hours the sec
tions were removed and the hormone content of the blocks was de
termined   The result of this experiment was that the sections placed
in the dark on blocks containing auxin *a* and *b* had lost 16 per cent
of the original amount of hormone, whereas in the experiment in
which the sections were placed in the light 42 per cent of the original
amount had disappeared from the blocks   If the sections were
placed on blocks containing hetero auxin, no growth hormone dis
appeared from the blocks, but 23 per cent was added to the amount
originally present in the block   This growth hormone obviously
came from the sections   If the sections contain growth hormone

FIG 5 The result of a peroxidase test showing the higher peroxidase activity
in the mesocotyl base (below) as compared to the mesocotyl tip

this hormone is auxin *a* or *b*   If this hormone is destroyed in the ex
posed sections, the result will be that less hormone is given off by the
exposed sections than by the sections in the dark   The amount of
hormone left over in the block on the basal cut surface is determined
by the amount destroyed in the block, and by the amount given off
bp the sections into the block   The amount of hormone destroyed in
the blocks is the same in the light as in the dark   This is shown by
Experiment 51,030, which is the same as the other experiment of
Table IX, but the coleoptiles were put overnight with their basal
cut surface in water after they had been cut off   By this procedure
sections free from hormone are obtained   From Experiment 51,029*a*

therefore it can be concluded that the hormone in the sections themselves is destroyed if the sections are exposed to light

Evidence in favor of the assumption that the inactivation of the growth hormone in plants that are exposed to light is also an oxidative process is as follows   Skoog (1935) showed that if a small amount of eosin was added to a growth hormone solution the hormone was inactivated if this mixture was exposed to the light   He showed that this was due to oxidation   Boas (1933) showed that if a seedling is infiltrated with eosin it is unable to show phototropic curvatures Boysen-Jensen (1934) showed that if roots are infiltrated with ery-

TABLE IX

Destruction of Auxin-A and B and Hetero-Auxin by Sections of Avena Coleophiles in Light and Dark

Temperature 23°C   Time 2 hrs   Twenty sections per block of 8 x 6 x 1 mm

No	Hormone	Concentra tion started with	Left over		Disappeared	
			Light	Dark	Light	Dark
					per cent	*per cent*
51 029*a*	Auxin-*a*	9 0	5 5	7 7	42	16
		7 7	4 3	6 4		
	Hetero-auxin	6 0	—	8 0	—	*−23*
		6 4	—	7 1		
51,030	Auxin-*a*	10 2	5 8	4 5	43	48
		10 3	6 0	6 3		
	Hetero auxin	12 1	8 0	8 8	*27*	*18*
		11 3	9 2	10 3		

throsin (yellow eosin) they fail to respond geotropically   He was able to show that eosin treated roots give off a smaller amount of growth hormone than normal ones   .

If in Table IX the figures for the destruction of hetero-auxin are compared with the ones for auxin-*a*, we find in the dark a destruction of 18 per cent for hetero-auxin to 48 per cent for auxin-*a* and *b*   In the light these figures are 27 per cent for hetero-auxin to 43 per cent for auxin-*a* and *b*

Table X shows the same for *Raphanus* sections   If the sections are exposed and the blocks contain auxin-*a* and *b* the amount of hormone left over in the blocks is even more than in the dark   In ex-

planation of this fact is lacking as yet   One thing, however, is shown clearly in these experiments, *viz* that the percentage of hormone which disappeared from the blocks is in every case larger for auxin *a* and *b* than for hetero auxin

The fact that hetero auxin is more difficult to destroy than hormones of the type of auxin *a* is not surprising if the results recently obtained by Kogl and Kostermans (1935) are considered [4]   They measured the activity of hetero auxin as compared to the auxins *a* and *b* and found that the activity of hetero auxin is about half that of

TABLE X

Destruction of Auxin A and B and Hetero Auxin by Sections of Raphanus Hypocotyls
Temperature 23°C   Time 4 hrs   Twenty sections per block of 8 x 6 x 1 mm

No	Hormone	Concentration started with	Left over		Disappeared	
			Light	Dark	Light	Dark
					per cent	*per cent*
51 025	Auxin-*a*	8 0	6 1	4 2	36	50
		—	5 0	4 0		
	Hetero-auxin	10 5	6 3	6 5	34	30
		—	7 5	8 1		
51 026	Auxin-*a*	8 2	6 6	5 3	15	40
		7 3	—	4 3		
	Hetero-auxin	6 6	—	7 4	—	−10
		6 7	—	7 1		
51 028	Auxin *a*	8 9	4 2	3 6	46	64
		9 3	5 5	3 0		
	Hetero auxin	8 7	5 3	5 6	23	20
		7 4	7 1	7 4		

auxin *a*   When they took the molecular weight into account, the difference was still larger   The "molecular activity" of hetero auxin is 3 75 times smaller than that of auxin *a*   This means that in order to get the same curvature with hetero auxin as with auxin *a* 3 75 times more molecules of the former substance are required   Assuming, for instance, that in the process of destruction 1 molecule of

[4] After this article was in press, it came to my attention that Thimann and Went (1934) had shown that auxin *b* is more easily inactivated by hydrogen peroxide than hetero-auxin (p 459)

hetero-auxin is as easily oxidized as 1 molecule of auxin-*a*, it is clear that under the same conditions the activity of the auxin-*a* will decrease 3 75 times as fast as that of the hetero-auxin

10 The Light Growth Responses of the Avena Coleoptile —From studies by Van Dillewyn (1927), Went (1925), and a recent study by Haig (1935) we know that two types of light growth responses can occur in *Avena* coleoptiles   One of them is called the tip response because it occurs only when the tip is exposed to light   Even a very small amount of light is able to produce a tip response   Went proved that amounts of light able to produce a tip response also decrease the amount of growth substance given off by the tip   He assumes very logically that this decreased amount of hormone given off is the direct cause of the tip response   Since in the present investigations the tips were cut off, tip responses should be excluded   It will be shown, however, in the next section that the formation of growth substance in the decapitated coleoptile (regeneration) is inhibited by small amounts of light in the same way as the production of growth substance in the tip   In a coleoptile stump growing on regenerated growth substance it must be possible therefore to produce a tip response even though the tip of the plant has been cut off   In the experiments described in the previous sections the regeneration has been suppressed by decapitations

Besides the tip response a base response can occur in the coleoptile To produce a base response the base (which means here any part of the coleoptile but the extreme tip) must be exposed to relatively high amounts of light   The base response under conditions of continuous exposure consists of two parts   (1) Almost immediately after the plants are exposed the growth rate decreases and about three-quarters of an hour after the exposure was started the growth rate has reached a minimum   (2) Then the growth rate increases again, but stays lower than before the exposure started   Koningsberger (1923) considers this increase in growth rate following on the growth inhibition only indirectly linked to the growth inhibition   According to him the latter is superimposed on the former one

It is not difficult to draw a comparison between this base response and the results obtained with growth substance curvatures in dark

and light [5] The first part of the base response, the inhibition, can be compared with the experiments (1*b*) and (3*b*) of the scheme   The inhibition is caused by the destruction of auxin *a* under influence of the light   The second part of the base response, the increase, can be paralleled with (6*b*) and (3*b*) of the scheme   The increase in growth rate (and increase in curvature) as it is measured in the intact plant (or plant with auxin *a* blocks) is the result of 2 antagonistic processes (1) The response to growth hormone itself is larger after a long exposure (as can be shown with hetero auxin curvatures (6*a*) and (3*a*) (2) Superimposed on this is the destruction of auxin-*a* under influence of the light   This confirms Koningsberger's (1923) point of view, which does not consider the light growth response as a whole, but discriminates strongly between the inhibition and the "anti reaction "

Besides the tip response and the base response a third type of response in *Avena* has been described (Tollenaar, 1923, Van Dillewyn, 1927)   This third type of light growth response is the so called "dark growth response" which occurs if the exposure of a plant which has been exposed to light for a long time, is stopped   The response is an increase in growth rate   A parallel between this response and the experiments (4*b*) and (1*b*) of the scheme can be drawn   This response is due to the increased response to growth hormone   It must be borne in mind, however, that this dark growth response has nothing to do with the bringing back of the plants into the dark because if the plants are continuously exposed they show the response too as can be shown by using hetero auxin instead of auxin *a* (6*a*)   By bringing back the plants into the dark the destruction of the auxin *a* stops, whereas the higher response to growth hormone lasts, which results in an increased growth rate in the intact plant and an increased curvature if it is an auxin-*a* curvature

11 The Regeneration and Its Influence upon the Growth Substance in Light and Dark Curvature —This section is more or less independent of the previous ones in this paper   If the tip of a coleoptile is cut off the ability of the coleoptile to produce growth hormone is not damaged beyond repair, because about $1\frac{1}{2}$ hours after the tip

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[5] In the meantime it has been proved that they are identical (Van Overbeek (1936))

has been removed the cells in the apical part of the stump start to produce the hormone   This is called in botanical literature the "regeneration of the physiological tip" or briefly the regeneration   Van der Wey (1931) showed that if the regeneration was suppressed by repeated decapitations the growth substance curvature was larger than if regeneration occurred   Regeneration therefore inhibits the growth substance curvature   Tsi-Tung Li (1930) investigated the effect of temperature upon the regeneration   According to his figures
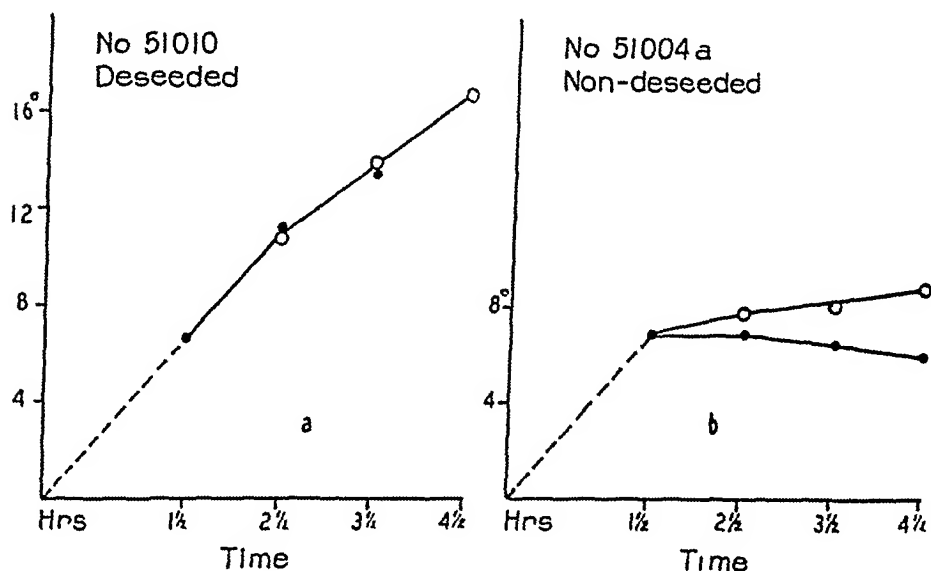
FIG 6   Growth substance curvatures with (b) and without (a) the interference of regeneration in dark and exposed hourly to 25 m c s   Abscissa, hours after application of growth hormone   Black dots, dark   Open circles, exposed to light   Mean values of twenty-four plants for each point

the regeneration starts 210 minutes after the tip has been removed at 15°C   At 25°C, however, this time is only 100 minutes   The newest contribution to the knowledge of regeneration has been made by Skoog (1937)   At a certain stage of development of the seedlings he removes the seeds   Such plants do not regenerate as he proves   In using these plants it is possible to study the growth substance curvatures without the interference of regeneration   In Fig 6 the difference in growth substance curvature with and without the interference of the regeneration can be seen   The abscissa represents the time in hours after the blocks containing auxin-a had been applied

to the twice decapitated plants   Fig 6b shows that after 1½ hours
the curvature in the dark (black dots) in non-deseeded plants does
not increase any more, but decreases   Fig 6a, however, shows that
in deseeded plants the curvature increases with time

What effect does light have upon the regeneration?   Tsi Tung Li
(1930) tried to answer this question by measuring the growth rate of
decapitated coleoptiles with a horizontal microscope   One set of his
plants had been exposed to 44 m c for 30 minutes   After about 140
minutes at 20°C and after 100 minutes at 25°C the growth rate in
creased, without showing a noticeable difference between the exposed
and non exposed coleoptiles   Tsi Tung Li concludes that "light
exerts no effect on the moment for the appearance of the physiological
tip"   This conclusion, however, is not justified because the growth
rate of the decapitated plants depends upon two factors at least   (1)
The amount of growth substance regenerated   (2) The response of
the plant to growth hormone   According to Sections 3, 7, and 10 of
this paper, the plants with which Tsi Tung Li worked were pre-exposed,
and, therefore, very probably had a higher response to growth hor-
mone than the non exposed controls   As will be shown below, in
three different ways it can be proved that light inhibits the regenera-
tion

1   As Gorter (1927) has pointed out, the positive curvature (which
is towards the block) appearing when plain agar blocks are put on one
side of decapitated coleoptiles, is due to the formation of growth sub
stance in the decapitated plants   If such plants are exposed to
amounts of light smaller than are required for base responses, the
positive curvature is smaller in the exposed plants than in the controls
Table XI shows the results   Plain agar blocks were put on the cut
surface of twice decapitated plants   1½ hours later the plants already
showed a small positive curvature   The plants were photographed
with a small amount of light (25 m c s)   This amount is sufficient
to produce a tip response and insufficient for a base response (Van
Dillewyn (1927))   1 hour after the photograph had been taken, and
hence 2½ hours after the plants had been decapitated and the agar
blocks put on, these plants show a curvature of only 0 5° whereas
the controls which had been kept in the dark all the time show a curva
ture of about 2°   This proves that regeneration is inhibited by light

even in such small amounts as reduce the amount of growth substance given off by the tip of the coleoptile (Went) and as produce a tip response only (Van Dillewyn)

2 If a set of plants to which a small amount of growth substance has been applied is exposed hourly to an amount of light of about 25 m c s , the growth substance curvature of these plants appears to be larger than those of non-exposed controls   The experiment is as follows   eight rows of twelve plants were decapitated twice and growth substance blocks of a concentration of 7° were applied   The plants were then divided into four groups of twenty-four plants each   One group was exposed (and photographed at the same time) 1½ hours after the blocks had been applied   After an hour this group was photo-

TABLE XI

Positive Curvatures of Twice Decapitated Avena Coleoptiles to Which Plain Agar
Was Applied on One Side   One Set of Plants Has Been Exposed to 25
M C S , 1½ Hrs after the Agar Was Applied

Curvature 1½ hrs after the agar was applied	1 1
	0 5
Same plants 1 hr later	0 4
	0 5
Curvature of non-exposed plants, 2½ hrs after agar was applied	1 8
	2 3

graphed again and another group which had *not been exposed before* was also photographed   Again an hour later the first group was photographed and so was a third group, which therefore had been in the dark for 3½ hours   The next hour the first group was exposed again as well as the last group which had not yet been exposed   In Fig 6b the open dots represent the first group, whereas the black dots represent the non-exposed controls   It is obvious that the curvatures of the plants which had been exposed are larger than those of the non-exposed ones   The explanation must be that the inhibition of the growth substance curvatures is less in the case where the plants are exposed, which point of view is justified by the next experiment (Fig 6a)   The same experiment as described above was repeated with plants which had been prepared according to Skoog's method

In such regeneration free plants exposure to 25 m c s does not have any effect upon the growth substance curvature

3 A direct proof that light inhibits the regeneration can be given as follows coleoptiles were decapitated and were exposed either to a large amount of light (first two experiments of Table XII) or to a small amount of light (last two experiments of Table XII) After about 4 hours the tips of the coleoptile stumps were cut off, and twenty of them were placed on plain agar blocks of $8 \times 6 \times 1$ mm for $2\frac{1}{2}$ hours The extraction of the hormone took place in the dark in order to eliminate possible transport and destruction differences between the exposed tips and the non exposed controls Later the blocks were tested on deseeded plants, because the amounts of hormone

TABLE XII

Amount of Regenerated Growth Substance Given Off by Top Sections of Decapitated Avena Coleoptiles in Dark and Light   Analysis on Deseeded Plants Photographed 5 Hrs after the Blocks Were Put on   Temperature 23°C

No	Amount of light	Amount given off	
		Light	Dark
50 920	500 m.c. during 2 hrs	2 5	5 4
		2 8	6 2
51 012	25 m c.s each hour (4×)	3 5	5 6
51 016	Same	3 2	5 4

given off by the sections are in general too small to be analyzed by the standard method In every case the amount of hormone given off by the exposed plants was less than the amount given off by the tips of non exposed coleoptile stumps

If Figs 2 and 3 of this paper are compared with Figs 1 and 4 a striking difference is obvious The curve of the latter figures starts at the origin (zero point) and is similar to the concentration curves published by Went (1928) and Van der Wey (1931) The curves of Figs 2 and 3, however, cross the abscissa This means that the curvatures measured are not proportional to the concentration of the hormone in the block Scores of other tests made at temperatures between 27 and 23°C showed the same Since the

deseeded plants, which do not show regeneration, show a normal curve an explanation of the abnormal curve may be the early start of the regeneration at higher temperatures   In favor of this point of view is the fact that exposed plants more than the non-exposed ones (Fig 2) show a curve which approximates the regular curve (starting at zero)   Against it, however, can be said that Went's experiments are carried out at 25°C   He used much smaller agar blocks though than the ones in these experiments, which may have an effect upon regeneration and growth substance curvature

<div align="center">III</div>

<div align="center">SUMMARY</div>

An attempt has been made to analyze the base response, one of the light growth responses of *Avena* coleoptiles, by means of growth substance curvatures   The decrease in growth rate (first part of the base response) after exposure to light does not show if hetero-auxin is substituted for auxin-*a* (Sections 5, 6, and 10)   This decreased growth after exposure very likely is due to an oxidative inactivation of auxin-*a* (Sections 8 and 9)   Hetero-auxin can be inactivated too but in a much lesser degree than auxin-*a* (Section 9)   The increase in growth rate following on the decreased growth (second part of the base response) is due to an increase in response of the plant to growth hormone which is independent of the type of hormone (Sections 1, 2, 7, 8, and 10)   Under conditions of continuous exposure to light, however, the inactivation of the auxin-*a* under influence of the light is superimposed on this increased response to growth hormone   This inactivation can be eliminated from the light growth response by replacing the auxin-*a* by hetero-auxin   More detailed information on this subject can be found in Section 10   A review of the experiments and their results can be obtained from the scheme in Section 8

In Section 11 it is shown that light inhibits the formation of growth hormone in the decapitated coleoptile (regeneration)   Very small amounts of light (25 m c s) inhibit the regeneration markedly

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A DESEEDED AVENA TEST METHOD FOR SMALL
AMOUNTS OF AUXIN AND AUXIN PRECURSORS

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INTRODUCTION

In 1927 Went isolated the growth promoting hormone, auxin, from
the tip of the *Avena* coleoptile, and worked out the now well known
Avena test method for its quantitative determination   By the use of
this method the chemistry and many phases of the physiological action
of auxin have been studied   In physiological work, however, the
amounts of hormone involved are frequently so small that quantitative
or even qualitative work has often been very difficult or impossible
In this article is presented a supplementary procedure with deseeded
Avena seedlings whereby smaller concentrations of the hormone not
detectable by the standard method can be quantitatively determined
By the use of this method it has also been possible to demonstrate
directly the existence of substances capable of being converted into
auxin by the plant   Some data relative to the presence of a precursor
of auxin in *Avena* and synthetic precursors of hetero auxin are in-
cluded

Description of Materials and Methods

1 The Standard Method —Since the deseeded method involves only changes in
technique, it need not be described in detail but rather in relation to the standard
method which will first be reviewed   Oats of the pure line *'Sieges Hafer* of
Svalöv are dehusked, soaked in water for 2 hours and laid out on wet filter paper
in a moist dish in the dark room kept at 25 C 90 per cent humidity, and free
from any phototropically active light or toxic gases   The following day the seeds
are planted in individual glass holders (Fig 1) and placed with the roots in water,
twelve plants in a tray   2 days later when the coleoptiles are about 3 to 4 cm
long, they are ready to be used for tests   The plants are decapitated, about 0 5
cm of the tips being removed and the primary leaves are pulled loose from the

base   The plants are then allowed to stand for 40 minutes, so that the upper portions of the coleoptiles will become largely free from hormone, and so that any individual plants that might show curvatures due to handling can be detected and removed   At this time small agar blocks of standard size containing the hormone solution to be tested are applied to the cut surface on one side of the coleoptiles   After 110 minutes of application the plants are photographed as shadow pictures on bromide paper   The curvatures produced by the unilateral application of the hormone can then be measured with a goniometer from the pictures   For a single test the mean value of the curvatures of one row of twelve test plants is used   A complete description of the technique is given by Went (1928) and also in some detail (1935a)

It has been established by van der Weij (1932) and by Thimann and Bonner (1932) that under the conditions of the standard test the curvature is proportional to the concentration of hormone in the blocks of a given size in the range of concentrations from 1 to 15 or 25 degrees depending on the daily variation in the maximum angle   The amount of hormone diffusing from the block into the plant in the given 110 minutes of application, however, varies with the size and hormone concentration of the agar block in two respects   through the difference in contact surface and through the decrease in concentration gradient during the time of application   The point is illustrated in the original experiments by Went in which small agar blocks were used and from which 90 per cent of the hormone disappeared during the test, and those by Thimann and Bonner in which standard (eight times larger) blocks were used and from which only about 15 per cent disappeared in the same time   Thus in the former experiments the curvatures were indeed roughly a linear function of both the concentration and the amount of hormone applied, and therefore, also with these blocks smaller amounts of hormone could be determined   For other reasons, however, such as ease of manipulation and especially less susceptibility to change in volume by drying out, larger blocks are superior and have been adopted for standard tests   In this work in accordance with the specifications and units defined by Dolk and Thimann (1932) 1 5 per cent agar blocks of the dimensions $\frac{8}{3} \times \frac{10\ 7}{4} \times 1\ 5$ mm $= 10\ 7$ ml have been used   The amount of hormone in one such block that will give a curvature of $1°$ under the above conditions corresponds to 0 4 A E (*Avena Einheiten*) and is therefore about 1×10^{-8} mg

It should be noticed that under the above conditions only a fraction of the hormone applied has been utilized by the plant in the test, which must be completed within 2 5 hours after decapitation   After this time synthesis of auxin is resumed in the new physiological tip of the coleoptile, and since regeneration takes place especially on the side not in contact with the agar block (see page 330), the rate of bending of the coleoptile no longer remains proportional to the concentration of auxin applied   Hence changes in the procedure have been introduced Most notable are those described by van der Weij (1931), who has developed special tools for decapitation and has introduced the use of a second decapitation

1 hour after the first one   This technique has also been used in the experiments below   It has been shown by Dolk (1926) that successive decapitations at 2 hour intervals prevent the regeneration of auxin in the plant   In accordance with this effect the double decapitation delays regeneration of auxin, and makes it possible to work with a larger number of plants   But the actual time of application of the blocks cannot be markedly increased by this method

2 *The Deseeded Method* —It has long been assumed, and will be shown here, that the auxin synthesized in the tip of the coleoptile is derived from a precursor transported from the seed   By deseeding, the
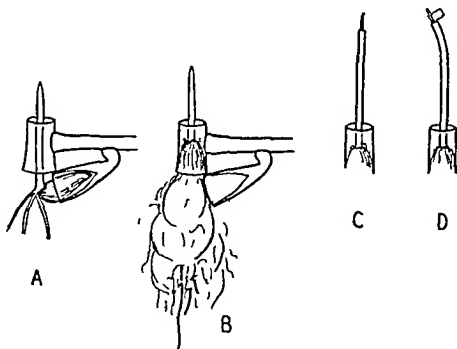

FIG 1 Diagrams of steps in deseeded test showing plant *A*, ready to be de seeded *B*, deseeded and replaced in holder, *C*, after second decapitation, *D*, after application of blocks and ready to be photographed

source of auxin is removed, thus regeneration is prevented, and as a result more sensitive test plants are obtained   Hence, the changes in the standard procedure, briefly illustrated in Fig 1, are as follows   on the 2nd day after germination, when the coleoptile is about 1 5 cm long, the plant is taken from the holder, and the entire seed, with the exception of the lower half of the scutellum, is removed   A small piece of cotton is wound around the lower portion of the seedling, which is then reinserted in the holder with a pair of bent forceps (eye forceps) The cotton serves both to hold the plant and to insure a good water supply to the coleoptile   12 to 18 hours later, as in the standard pro

cedure, the plants are ready to be used for tests   Photographs may be taken singly or repeatedly at intervals at any time up to 5 or 7 hours after application of the blocks   A comparison of the curvatures obtained with this method and with the standard method is shown in Fig 4

Physiological Factors Affected by Deseeding

The effects of the removal of the seed on the subsequent development of the coleoptile are manifold   They will be discussed here mainly in relation to the growth response to auxin

1 Effect of Deseeding on Linear Growth —The effect of deseeding on linear growth has been studied by Went (1935b)   He finds that the growth rate in deseeded plants is decreased about 40 per cent, and that this decrease is due to the lack of two factors, auxin and food   Furthermore, the application of high concentrations of auxin to intact deseeded plants causes about the same percentage increase in growth as in normal plants, but in deseeded plants the growth is more limited to the upper part of the coleoptile   In accordance with this behavior is the fact that in deseeded, decapitated coleoptiles, much sharper apical curvatures are obtained by the unilateral application of the hormone, *i e*, the basal parts do not grow and thus remain straight even after long times of application of the hormone

The effect of deseeding on regenerative linear growth is shown in Fig 2, in which is represented one of five similar experiments   Measurements of growth were made with a horizontal microscope on plants which had been marked with India ink into three zones   Each curve is the mean of three or four plants   The ordinates represent the sum of the total increase in length of two zones, which include almost the entire coleoptile in the decapitated plants and the corresponding two zones in the intact plants   Measurements were started 1 hour after decapitation   Curves I A and B represent intact and decapitated control plants respectively   Curves II and III A and B represent corresponding sets of plants which had been deseeded 1 and 10 hours respectively before the time of decapitation   From these curves the following facts are clear   By deseeding, growth is decreased both in intact and in decapitated plants   The amount of decrease is a function of the time after deseeding   In plants that were deseeded only 1

hour before decapitation, the regenerative growth in decapitated plants is only slightly reduced   In plants that were deseeded 10 hours before decapitation, regenerative growth is markedly inhibited   However, how far the decrease in regenerative growth depends on the lack of auxin alone or in addition on the lack of other factors, food, cannot be safely determined from the above curves
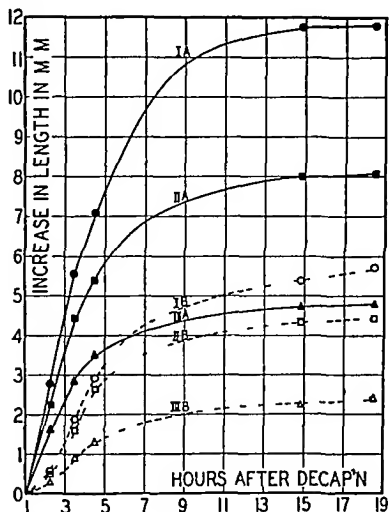

FIG 2 Linear growth of normal and deseeded intact (solid line A) and decapitated (broken line, B) plants   Curves I normal, curves II and III deseeded 1 and 10 hours before decapitation respectively   Measurements started 1 hour after decapitation

2 *Effect of Deseeding on Geotropic and Auxin Curvatures* —It will be shown that curvature growth, i e the difference in the relative growth of the two sides of the coleoptile, in deseeded and non-deseeded plants under the conditions described for the deseeded test, is independent of food and is a function only of the amount of auxin present

ın the coleoptıle   The followıng three types of expenments demon-
strate this conclusıon

a Decrease ın Auxın Synthesıs after Deseedıng —Begınnıng on the
2nd day after germınatıon, plants were deseeded, thırty-sıx at a tıme,
at successıve ıntervals   Then at a gıven tıme, 3 mm long coleoptıle
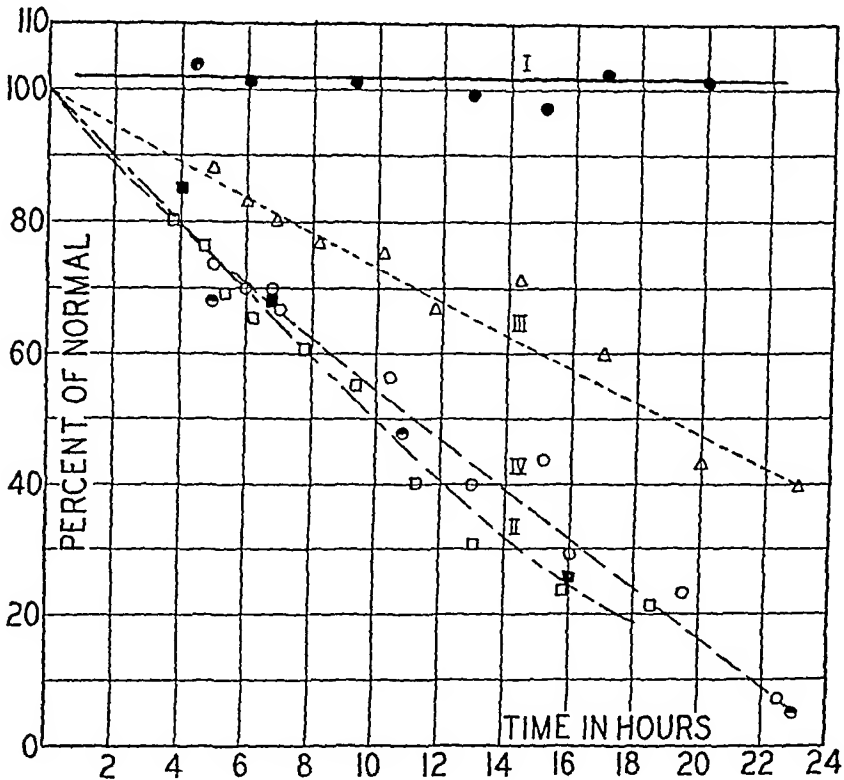tıps were cut off and placed on standard agar blocks, 15 tıps per 12

FIG 3 Effect wıth tıme of deseedıng on sensıtıvıty to auxın, I, synthesıs of
auxın ın tıps, II, geotropıc response ın ıntact, III, ın decapıtated, IV, coleoptıles
All values are expressed as per cent of normal controls

blocks, for 2 hours   The amount of hormone dıffusıng out from the
dıfferent sets of tıps was determıned by the standard *Avena* method
All the blocks were tested at the same tıme wıth twenty-four test
plants for each set of deseeded tıps and forty-eıght plants for the con-
trols   The amount of hormone produced by deseeded plants expressed
as per cent of that produced by normal plants ıs plotted agaınst tıme
after deseedıng ın curve II of Fıg 3, whıch represents the mean values

of several experiments   The curve shows that there is a continuous decrease in the rate of auxin synthesis after deseeding, and at least for a considerable period this decrease is closely a linear function of time

b Sensitivity to Auxin —The relative sensitivity to auxin of deseeded plants at different times after deseeding, *i e* the capacity to produce curvatures in response to auxin applied unilaterally in blocks of moderate concentrations (5 to 20°) for 110 minutes as in the standard test, and compared with the curvatures produced by the application of the same concentrations of hormone in normal plants of the same group, is shown in curve I of Fig 3   In this curve, which represents the mean values of many experiments with over 600 plants, the curvatures of deseeded plants, expressed as per cent of the curvatures obtained in corresponding control plants, are plotted against time after deseeding   It is clear that the sensitivity of deseeded plants is at least as great as that of normal plants   Only when the interval between deseeding and the test is made very long (not included in the graph) and also, which is probably more important, when the plants are deseeded in a very early stage of development, is there evidence of a distinct decrease in sensitivity   It appears from the curve that the sensitivity of deseeded plants may be slightly higher than that of normal plants   If this increase be real, it is probably due to the fact that in normal plants regeneration has begun less than 2 5 hours after the second decapitation (see also below)   However, it should be pointed out that for moderate concentrations of hormone, this difference is so small that unless a very large number of tests are made, it is well within the experimental error

c Decrease in Geotropic Curvatures in Deseeded Plants —It has been shown by Dolk (1926) that the geotropic curvature in normal intact and decapitated coleoptiles is controlled by the amount and relative distribution of auxin in the organ   Coleoptiles not producing auxin, as for example freshly decapitated plants, show no geotropic response   But similar coleoptiles to which auxin has been applied over the entire cut surface produce geotropic curvatures which are proportional to the concentration of hormone applied   Furthermore, the amount of hormone obtainable by diffusion from the upper and lower sides of geotropically stimulated coleoptiles is proportional respectively to the growth of the two sides   Since it was shown in section 2 *b* that the

sensitivity to auxin is not decreased by deseeding, it can be said with fair certainty that the relations between auxin and geotropic curvature, established by Dolk for normal plants, hold also in deseeded plants   Thus in conjunction with the experiments of sections *a* and *b*, a large number of determinations were made of the relative geotropic response in deseeded plants   At definite times after deseeding, de-

Fig 4 A

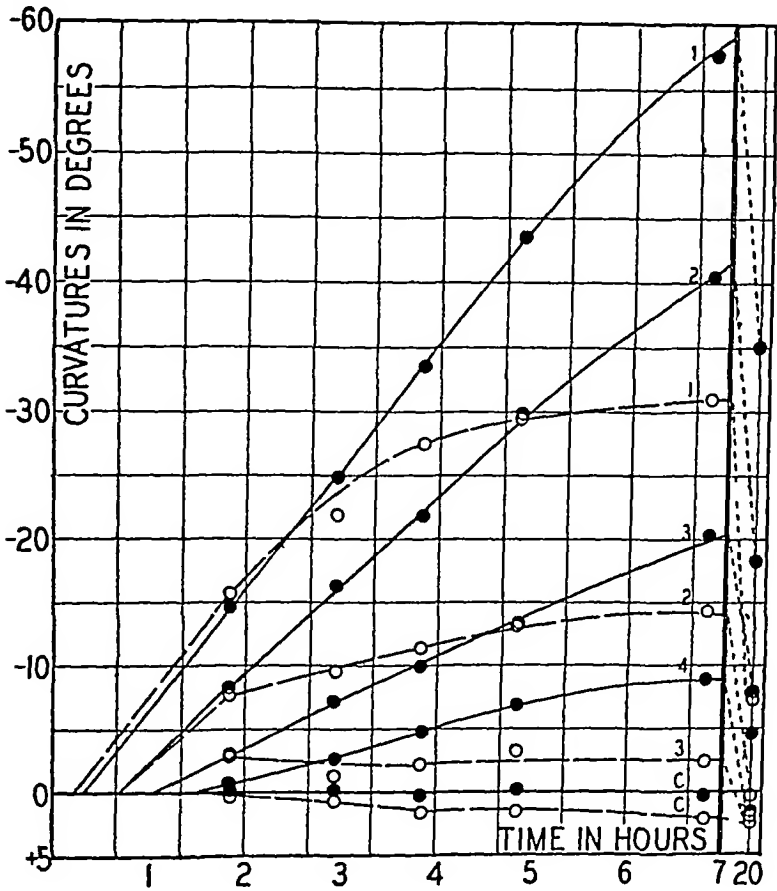seeded and normal plants of the same group were placed horizontally The geotropic curvatures produced in a given time, 1 hour for intact plants and 4 hours immediately after decapitation for decapitated plants, were measured from photographs taken at the end of the specified times   The curvatures of deseeded plants expressed as per cent of those of normal plants and plotted against time after deseeding are

shown in curves III and IV of Fig 3, representing intact and decapitated plants respectively The curves demonstrate that in both intact and decapitated coleoptiles of deseeded plants there is a decrease in geotropic response proportional to the decrease in auxin synthesis Thus, if the plants are decapitated about 15 hours after deseeding, subsequent regeneration of auxin is nearly completely prevented
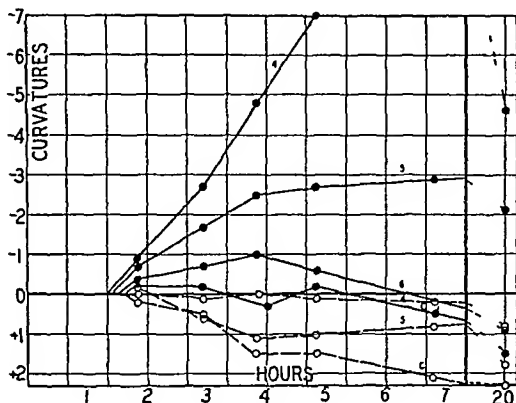

FIG 4 B

FIGS 4, *A* and *B* Comparison of deseeded (solid lines) and standard (broken lines) tests for different concentrations of hormone

Curves	1	2	3	4	5	6	and	C
correspond to concentrations of	1	1/2	1/6	1/18	1/54	1/108	and	0

respectively

From a consideration of the data in Fig 3 as a whole it is possible to draw one further conclusion Since the decrease in geotropic curvature in deseeded plants is independent of the sensitivity of the coleoptiles and thus depends only on the decrease in auxin synthesis, and since the relative decrease in auxin synthesis in regenerating decapitated coleoptiles is very nearly the same as that in decapitated tips and proportional to that in intact tips, it follows that the mechanism

of auxin synthesis in the tip of the intact coleoptile and the mechanism of regeneration of auxin in the new physiological tip of the decapitated coleoptile are identical

3 Comparison of the Deseeded and the Standard Tests —We are now in a position to consider the relations between the deseeded and the standard tests in terms of physiological factors affected by deseeding The curves in Fig 4 *A* and *B*, obtained in one of six experiments with practically identical results, represent curvatures in degrees plotted against time in hours of unilateral application of hormone of different concentrations to deseeded and to normal plants   For the sake of clearness the lower concentrations are plotted separately on a larger scale in *B*   In this experiment the deseeding was done 15 hours before the time of the first decapitation   Photographs were taken after 110 minutes of application as in the standard test and then at successive intervals

a Regeneration and Temperature Effects —By comparing each continuous line (deseeded plants) with its corresponding broken line (normal plants), it becomes clear that for moderate concentrations of hormone above 3°, the curvatures are the same in both tests for the first 2 hours   However, after this time regeneration sets in   As a result, in normal plants the rate of bending is decreased, so that the curvatures recede, remain constant, or continue to increase at a slower rate, according to the concentration of hormone applied   In deseeded plants, where regeneration is practically completely lacking, the curvatures continue to increase linearly with time for several hours, or if the concentration of hormone is smaller, until the supply of auxin from the blocks has been largely depleted   As a matter of fact, in normal plants a small amount of regeneration takes place earlier than 2 5 hours after the second decapitation   Thus in the standard test, when blocks of low concentrations are applied, practically no curvatures will appear, when blocks of very low concentrations or pure agar blocks are applied, positive curvatures, *i e* in the direction towards the blocks, will occur   The cause of these positive curvatures will be clear from a consideration of the precursor of auxin, which diffuses out into the agar blocks and is not immediately converted into auxin Also in deseeded plants small positive curvatures may be obtained by the unilateral application of pure agar blocks, but the effect is much

less than in non deseeded plants   Thus for determinations of small concentrations of hormone, deseeded plants are relatively even more sensitive than for higher concentrations   This additional sensitivity appearing already within the first 2 hours of application is clearly brought out in Figs 5 and 6, in which the curvatures obtained in two experiments as determined by deseeded and standard tests are plotted
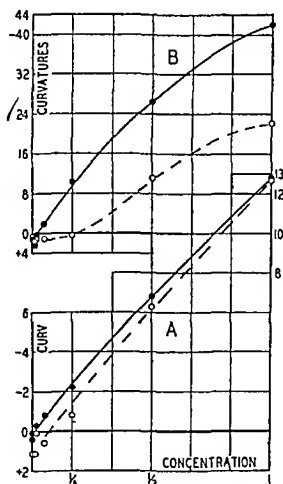
FIG 5 Comparison of curvatures obtained by deseeded (solid line) and standard (broken line) tests plotted against concentration of hormone applied   A, photos taken after 110 minutes B, after 390 minutes application

against concentration of hormone applied   The curves obtained by the use of normal plants intersect the abscissa some distance away from the origin, whereas the curves obtained with deseeded plants come very close to the origin   The distance from the origin to the point of intersection of the curve with the abscissa for normal plants increases very rapidly with increase in temperature as does regeneration   But the question whether regeneration is the entire cause of the

decreased sensitivity or whether there is in addition a small destruction
of auxin by the plant (see Van Overbeek (1936)) must be left open,
because the curvatures involved are so small that the two effects can-
not be clearly differentiated in these experiments   At higher tem-
peratures (27°) autotropism, perhaps due to regeneration, becomes
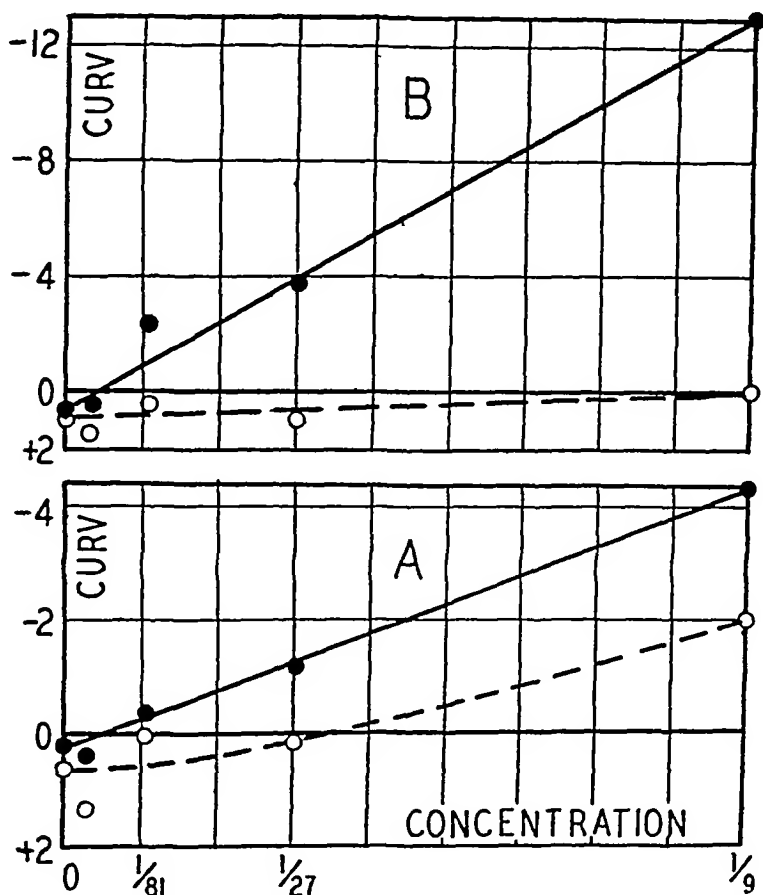
FIG 6 Experiment similar to Fig 5 showing lower concentrations on a larger
scale   A, photos taken after 110 minutes, B, photos taken after 300 minutes
of application

noticeable also in deseeded plants, so that after about 4 hours the
linear relationship between curvatures and time of application becomes
less pronounced

    b *Less Physiological Aging* —Also contributing to the higher sensi-
tivity of deseeded plants, especially long times after decapitation, is

the decrease in physiological aging Du Buy (1933) and Went (1935b) have shown that decapitated coleoptiles prevented from synthesizing auxin and not supplied with auxin for several hours, gradually become less sensitive to subsequently applied auxin This effect, "physiological aging," is due to the increase in thickness and loss of plasticity of the cell walls By deseeding, the materials for secondary cell wall formation are largely removed, and the walls of the coleoptiles remain thin and plastic even though the actual amount of auxin in them is less than in normal plants

c Limits for Concentrations and Amounts of Hormone —In general it can be said that with the deseeded method about ten times as small concentrations can be determined as can be detected with the standard test This fact is readily understood, if we consider that in the standard test only 15 per cent of the auxin passes from the agar block into the plant, whereas in the deseeded test, if photographs are taken after about 5 hours, nearly all the auxin in the block is utilized For example, if two blocks containing very low concentrations are placed one on top of the other on a deseeded test plant about twice as large curvature is obtained as by a single block In the standard test on the other hand, van der Weij found the curvature to be independent of the size of the block However, it is clear that in the deseeded as in the standard test there is a distinct limit to the concentration of auxin that can be detected If the limit for the standard method is taken as 1×10^{-8} mg per block, then the limit for the deseeded method is about 1×10^{-9} mg per block

d Effect of Light —Since white light has a marked effect on the growth of the *Avena* coleoptile, its influence on curvatures when successive photographs are taken must also be considered These experiments, which have a bearing on the light growth reaction, have been done by Van Overbeek, and the data given in Fig 7 have been kindly contributed by him In the standard test the plants are not exposed until the end of the experiment In the deseeded test, however, it is often desirable to make a series of estimations of the curvatures of a given set of plants Van Overbeek (1936) has shown that the amounts of light necessary for taking photographs partially inhibit regeneration In accordance with this as shown by curves A, Fig 7, the increase in curvature with time obtained from a series of photo

graphs at consecutive intervals of a given set of normal plants is greater than that obtained from plants of the same group, but of which a different set of plants, not previously exposed to light, is used at each corresponding interval Curves B of the figure represent the identical
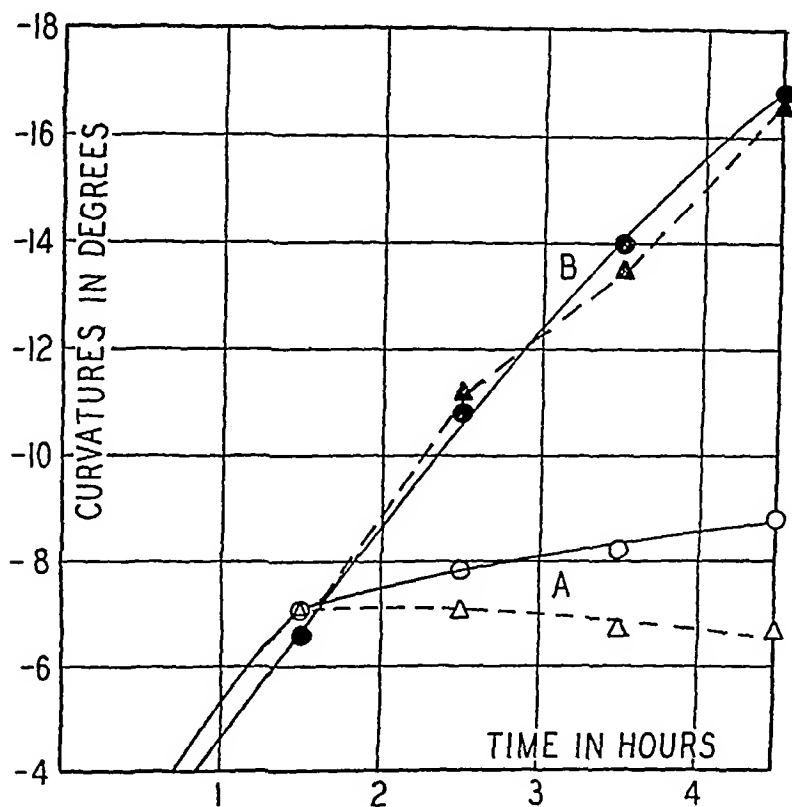
FIG 7 Effect of light on curvatures in normal (curves A, open points) and deseeded (curves B, solid points) plants Values represented by circles (solid lines) are from repeatedly exposed plants Values represented by triangles (broken line) are from different sets of plants of the same group and not previously exposed

experiment with similar plants deseeded 20 hours before the auxin was applied From the close agreement between the curvatures of successively exposed and not exposed plants, it is clear that the amount of white light necessary for photographing has no effect on the rate of bending of deseeded plants

Application of the Deseeded Test for Auxin Determinations

As illustration of the deseeded test, two examples will be given which include some data previously not easily obtainable

1 Determination of Auxin in Primary Leaves of Avena —Primary leaves of 4 day old plants grown in the dark room were pulled out of the coleoptiles and placed with their bases on agar blocks, twelve leaves per twelve blocks, for 2 5 or 4 0 hours The blocks were then tested on deseeded plants Control agar blocks on which no leaves had been placed were tested at the same time For comparison one standard test was also made The results of three experiments with

TABLE I

Auxin from Primary Leaves

Exp No	Time of		Mean curvatures from		
	Diffus on into block	Application in deseeded test	Blocks with diffusate in test		Plain agar in deseeded test
			Deseeded	Standard	
	h s	*hrs*			
1	2 5	8 0	−3 9	0 0	+0 1
2	4 0	6 0	−4 5	—	+0 4
3*	3 5	5 0	−2 3		+0 7
			−2 1		
			−2 6	+0 7	
			−2 8	+0 7	

* In Exp 3 18 leaves per 12 blocks were used but values are divided by 1 5 to give degrees in terms of 12 leaves per 12 blocks

leaves grown under different conditions, and which are therefore not comparable with each other, are summarized in Table I With the deseeded test the presence of auxin is clearly demonstrated, whereas with the standard test none can be found, and, in fact, has been thought to be absent Furthermore, the close agreement between the values obtained in different tests of the same experiment shows that the method gives quantitative results

2 Determination of Auxin in Coleoptile Sections —It has been shown by Thimann (1934) by the use of chloroform extractions from a large number of plants that in coleoptile sections auxin is present in small amounts This finding has been confirmed by placing 0 3 cm long

coleoptile cylinders on agar blocks, which were subsequently tested with deseeded plants   As shown in Table II, the amounts of auxin obtained are large enough to give quantitative measurements   Comparable experiments with the standard test give at best only a perceptible curvature

For a computation of the actual amounts of hormone obtained in the above experiments, it is best to compare the curvatures directly with those obtained in similar tests with successive dilutions of an

TABLE II

Auxin from Coleoptile Sections
3 Mm Long Sections Placed 20 Sections Per 12 Blocks

Exp No	Time of diffusion into block	Mean curvatures from			Time after application of blocks to 2nd photo
		Diffusate blocks		Plain agar 2nd photo	
		1st photo	2nd photo		
	hrs				*hrs*
1*a*	4 0	−2 3	−5 9		
b		−2 7	−6 2	+1 2	5
2	3 2	−1 2	−4 8	+1 4	8
3	3 5	−0 4	−3 4	+1 8	7
4*a*	4 0	−1 6	−5 7		
b		−1 3	−3 5	+1 6	10
5*a*	4 0	−1 0	−4 1		
b		−0 7	−2 7	+2 2	5
6*a*	4 0	−0 5	−3 0		
b		−0 5	−3 4	+2 6	9

auxin solution of known, relatively low concentration   From such a comparison it was estimated that the auxin obtained from the primary leaf is of the order of 0 05 A E per leaf per hour, and that from sections about 0 03 A E per section per hour   These amounts are only 5 or less per cent of the amount obtainable from the coleoptile tip per hour

The above experiments, although not carried out in detail, indicate the possibilities of the application of the deseeded test to work concerned with the presence and relative distribution of the growth hormone in plant tissues   They also bring out the fact that the high sensitivity of the deseeded test holds for auxins in general and is not

limited to synthetic hetero auxin, which was used above exclusively in the development of the method

Precursors of Auxin

Another use of the deseeded method and for which purpose it was originally designed is the detection of precursors of auxin

1 Demonstration of a Precursor of Auxin in the Coleoptile —It has been shown above that there is a limit to the concentrations of hormone that can be detected by the deseeded test   If the concentrations of hormone are so small that they will not cause distinct curvatures to appear within the first 5 to 6 hours after application, curvatures will not appear at any time later   Furthermore, it is well known that the transport of auxin in the *Avena* coleoptile is strictly polar in the direction from the tip towards the base   Even with the deseeded test, no detectable concentration of hormone has been found to be actively transported in the opposite direction   In accordance with this fact when coleoptiles are decapitated, the primary leaves removed, and agar blocks are placed over the entire cut surface of the stumps for 2 or more hours, and these blocks are then tested on deseeded plants, as expected, no curvatures are obtained within the first 5 or 6 hours of application   However, some time later, 10 to 20 hours after application, distinct negative curvatures are obtained   Results of some determinations are given in Table III   In the determinations made so far, the curvatures have varied considerably from one experiment to the next, but frequently the mean curvatures have been between 4 and 8°   The variability in the magnitude is to be expected, since the optimal experimental conditions must be governed by several factors, whose nature is as yet unknown

Perhaps a more striking way of demonstrating the precursor, which brings out the difference between it and auxin itself, is by the following arrangement   Sets of twenty coleoptile sections of given lengths, 5 or 3 cm , are placed with the bases either down or up, but all of a given set in the same direction, between two 12 times standard size agar blocks   After a given time, varying between 2 and 4 hours, the agar blocks are removed, cut each into twelve standard blocks, and tested on deseeded plants   The curvatures produced are measured

from photographs taken at intervals Results will not be given in detail, but the mean values of some fifteen separate determinations are presented by curves I, II, and III of Fig 8   These curves represent the curvatures plotted against time of application obtained from blocks previously applied to the basal (I) and apical (II) ends of sections and from control agar blocks (III) respectively   A comparison of the curves shows that the material diffusing out at the apical surface is different from that diffusing out at the basal surface in that it will only cause curvatures a long time after application   From the data

TABLE III

Precursor of Auxin from Coleophiles

Exp No	Time of		Mean curvatures from	
	Diffusion into blocks	Application in test	Apically applied blocks	Plain agar blocks
	hrs	*hrs*		
1	5 0	17	−2 6	
		20	−6 0	
		20	−4 0	
		20	−7 0	+0 4
2	3 to 4	24	−6 0	
			−7 2	
			−4 6	+0 3
3	1 5 or 3 5	16	−6 5	
			−4 9	
			−4 0	
			−5 9	+0 2
4	?	?	−1 6	
			−2 3	+0 8

in Table III and curve II of Fig 8 the conclusion is drawn that the growth promoting substance from the "apical" blocks is a precursor of auxin capable of being transported in the apical direction in the plant and capable of gradually undergoing a chemical change into auxin, whereas auxin itself can be transported in the plant only in a basipetal direction

The Relation between Positive Curvatures and the Precursor —It was shown above that by unilateral·application of plain agar blocks to decapitated plants small positive curvatures are produced   As early

as 1927 Gorter pointed out that these curvatures are not due to growth inhibiting substances, but are correlated with the regeneration of auxin in the new physiological tip   Why and by what mechanism regeneration is affected has not been made clear   From a determination of the amount of regeneration in coleoptiles with and without agar blocks, and from a consideration of the precursor of auxin, these questions will be answered   About 150 plants were decapitated   To half this
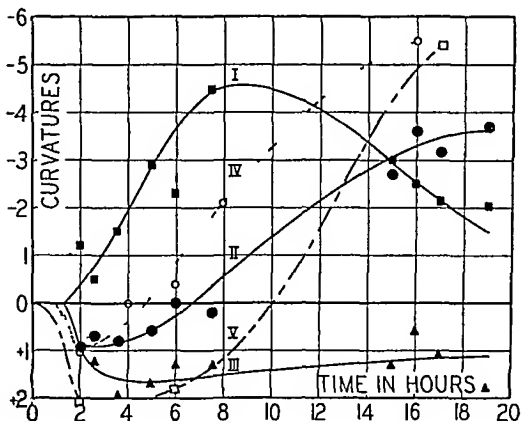

FIG 8 Illustration of curvatures obtained in deseeded plants by the application of blocks containing auxin from coleoptile sections (curve I), precursor of auxin from coleoptile sections (curve II) pure agar (curve III), tryptophane (curve IV) and indolethyl amine (curve V)

number plain agar blocks were applied to the entire cut surface of the coleoptiles immediately after decapitation, the other half was used for controls   Between 2 and 3 hours later 1 5 to 2 0 mm long apical sections were cut off and placed on agar blocks, twenty four sections per twelve blocks, for 2 hours   The amount of auxin produced by the sections was determined by testing these blocks on deseeded plants   The results (Table IV) show that in the apices of plants on which agar

blocks had been placed, the production of auxin was significantly less than in the controls without agar blocks Furthermore, tests of the agar blocks which had been placed on the apical surfaces showed no trace of auxin, but on the other hand indicated the presence of the precursor The mechanism of the formation of positive, differential regeneration, curvatures is therefore as follows On the side of the coleoptile in contact with the agar block a considerable fraction of the precursor of auxin diffuses out into the agar block and will not be immediately converted into auxin On the opposite side of the coleoptile precursor accumulates and is converted into auxin The relatively larger auxin production on this side makes possible a corresponding increase in growth, which causes a positive curvature

TABLE IV

Regeneration in Decapitated Coleoptiles with and without Agar Blocks Applied to the Cut Surface

Time of application of blocks	Mean curvatures with mean error from sections previously		Difference in degrees
	With blocks	Without blocks	
hrs			
3 0	−4 1 ± 1 2	−9 4 ± 1 2	−5 3 ± 1 7
2 5	−2 8 ± 0 8	−9 8 ± 1 2	−7 0 ± 1 4
2+	−4 9 ± 0 6	−7 1 ± 1 2	−2 2 ± 1 3

Curvatures from plain agar blocks +1 4 ± 0 3

2 Precursors of Hetero-Auxin —Since the chemistry of the auxin occurring in the *Avena* seedling is exceedingly complicated, because of the complex structure and instability of the active substance, no data on the chemical nature of the precursor have been obtained However, independent evidence, the chemical nature of which is better understood, will now be presented in support of the conclusions drawn from the experiments in section 1

It has been shown by Thimann that chemically pure tryptophane will not produce curvatures when tested by the standard *Avena* method But when tryptophane is applied to sections in solution, it will promote elongation Furthermore, from experiments on the synthesis of hetero-auxin by *Rhizopus suinus* Thimann (1935a) has

shown that tryptophane is a precursor of hetero auxin   The mecha
nism of the transformation is an oxidative deamination and decar
boxylation

What happens when very dilute tryptophane blocks are applied
unilaterally to deseeded plants?   It is evident from curve IV of Fig 8
that tryptophane behaves in exactly the same manner as the precursor
from the plant, curve II, with the exception that the effect of trypto
phane is greater   By choosing the proper concentration of trypto
phane, the same type of curve can also be obtained with normal test
plants, i e the amounts of hetero auxin formed from tryptophane are
large enough to more than balance the effects of regeneration and
autotropism, and may, in fact, cause the plants to be in a state of
active bending for more than 36 hours   It has been shown by Kogl
and Kostermans (1935) that indolpyruvic acid has auxin activity
Thus the possibility exists that this acid rather than hetero auxin
(β indolacetic acid) is formed from tryptophane   However, these
authors point out that the apparent activity of indolpyruvic acid
might likely be due to its breakdown into indolacetic acid   They
calculate that 1 per cent breakdown will account for the measured
activity

Another substance, indolethyl amine, kindly synthesized by Dr J
Koepfli, has been found to be very suitable for precursor experiments
This compound is completely lacking in growth promoting activity,
but in contact with the cut surface of the plant it can become activated
and will then produce curvatures, see Fig 8, curve V   It is superior
to tryptophane in that it is more slowly activated, does not contain a
carboxyl group which is possessed by all known active substances, and
its only active degradation product is hetero auxin   All lower degra
dation products are known to be inactive   It has further been found
that by placing agar blocks containing either tryptophane or β indol
ethyl amine in contact with the apical surfaces of a large number of
coleoptile cylinders for a short time they become activated, so that
when the blocks are subsequently applied to test plants, they will
cause large auxin curvatures, which start to appear almost immediately
after application

The experiments described in this section, although dealing with
substances evidently not identical with the precursor of auxin obtained

from *Avena,* nevertheless lend strong support for the evidence given above for its existence and behaviors They show that the delayed curvatures obtained above are due to an activation through chemical changes in the substances applied, and are not merely the result of any possible differences in the rates of transport in the plant of these substances and the auxins The activation of the precursor takes place most likely in the agar block in contact with the plant or extra-cellularly at the cut surface, since if the activation took place exclusively intercellularly, active substance would not be recoverable in the apical blocks Other experiments, not described here, exclude the possibility of activation through bacterial action Considerable evidence indicates that in the case of the synthetic precursors of hetero-auxin the nature of the activation process is fairly certainly an oxidative deamination How far the relationship existing between these synthetic precursors and hetero-auxin can be extended to explain the relationship between the precursor and auxin in the plant is as yet a speculative matter A more complete study of the chemical nature, specific physiological activities of the precursor of auxin, and the mechanism of its transport and activation in the plant is in progress

It has been pointed out by Thimann (1935*b*) and by Haagen-Smit and Went (1935) that a clear distinction must be made between true auxins and such substances as may promote growth but are not capable of being polarly transported in the plant It has further been shown by Michener (1935) that auxins must be distinguished from such substances as may indirectly affect the physiological activity of auxin in the plant In addition to these two groups must now also be considered a third group of substances capable of becoming activated by the plant into true auxins

<center>SUMMARY</center>

The main results presented in this article may be summarized as follows

1 A test method with deseeded *Avena* seedlings for small concentrations of auxin and precursors of auxin has been described

2 This method makes possible quantitative determinations of about ten times as low concentrations of hormone as can be obtained with the standard method (*a*) Through an increase in the time of the

test, so that nearly all the hormone applied can be utilized   (b) Through an increase in sensitivity of deseeded plants to unilaterally applied small concentrations of hormone

3 The effect of deseeding in relation to curvature growth is primarily the prevention of auxin regeneration through the removal of the material for auxin synthesis, and in addition the prevention of physiological aging

4 The mechanism of auxin synthesis in the tip of the coleoptile and the mechanism of auxin regeneration in the new physiological tip have been shown to be identical

5 The application of the deseeded method is illustrated by determinations of auxin in primary leaves and coleoptile sections of *Avena* seedlings

6 The deseeded method has been used as a test method for precursors of auxin obtainable from the coleoptile and from other sources The method further makes possible a distinction between auxins and these substances which may become activated by the plant

7 Evidence for the existence of a precursor of auxin in the plant is given (a) indirectly by determinations of the decrease in auxin synthesis in deseeded plants   (b) Directly by its isolation from the plant

8 Precursors of hetero auxin are demonstrated, their chemical nature and activation are briefly considered

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INACTIVATION OF PEPSIN BY IODINE AND THE ISOLATION OF DIIODO TYROSINE FROM IODINATED PEPSIN

By ROGER M HERRIOTT

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The previous work of the writer on the acetylation of pepsin (1, 2) points to a close relationship between the tyrosine of pepsin and the proteolytic activity of the protein molecule An acetyl derivative of pepsin was prepared which had 60 per cent of the activity of pepsin, contained three labile acetyl groups, and gave a tyrosine phenol color value lower by three tyrosine equivalents than that of pepsin For these and other reasons it seemed probable that the labile acetyl groups were attached to the phenolic hydroxyl groups of tyrosine in pepsin Attempts to isolate an acetylated tyrosine were unsuccessful due probably to the lability of the acetyl radical In the hope of obtaining direct and decisive evidence concerning the rôle played by tyrosine in pepsin it was decided to study the action of iodine on pepsin Iodine is supposed to react with the benzenoid part of tyrosine in proteins (3, 4) and since this type of iodine linkage is known to be relatively stable, it seemed likely that an iodine tyrosine compound could be isolated from iodinated pepsin

Under certain conditions solutions of native pepsin readily absorb iodine and the proteolytic activity decreases gradually Pepsin is practically inactive when the number of iodine atoms per molecule of pepsin is 35–40 There is no appreciable oxidation of pepsin or of glycyl tyrosine by iodine under the conditions used for iodination The rates of iodination of pepsin and of glycyl tyrosine are affected by a variation of pH in a like manner The effect of pH is nearly identical with that already noted for acetylation of these two materials

Completely iodinated pepsin was hydrolyzed and the products of hydrolysis containing iodine were fractionated A solution containing

82 per cent of the total iodine was obtained from the iodinated pepsin
The analyses of this solution were similar to those of a solution of
diiodo-tyrosine   Also present in this solution was a dark material
which prevented crystallization of the diiodo-tyrosine and which
could be removed only with difficulty and at the expense of the yield
of crystalline diiodo-tyrosine   The yield of crystalline diiodo-tyro-
sine finally obtained represented 53 per cent of the original iodine

That the iodine is attached to the tyrosine in the intact iodinated
pepsin protein is further indicated from the titration curve of the
iodinated protein   The groups in pepsin which titrate between pH
10 0 and 12 0, in the iodinated pepsin titrate between pH 8 0 and 10 0
These are probably the phenolic hydroxyl groups of tyrosine

<div align="center">EXPERIMENTAL RESULTS</div>

Conditions for Iodination of Pepsin

Many experiments were performed to determine the effect of the
numerous variables on the iodination of pepsin   The results of some
of these experiments are dealt with in detail in this paper but a large
part of this work has been omitted   It will suffice, however, to say
that the most reproducible and apparently uncomplicated results
were obtained by observing the following conditions   the concentra-
tion of free or molecular iodine in the reaction medium should not
be over one-tenth normal, the protein concentration is optimal at
2–5 mg protein nitrogen per ml and the pH kept at 5 0–6 0   The
temperature seems to affect only the rate of iodination   Alcoholic
iodine can be used in place of iodine-potassium iodide solution with-
out any apparent difference in the result unless the alcohol content
of the final solution rises above 10–15 per cent in which case the
temperature should be kept nearly 0°C and the time the protein is
in contact with this medium kept at a minimum

Effect of Iodine on the Proteolytic Activity

Under the conditions outlined above for the iodination of pepsin,
the specific activity of pepsin gradually decreases as iodine is intro-
duced into the protein molecule   Fig 1 shows the effect graphically
The specific activity is reduced practically to zero when the iodine
content is 35–40 atoms of iodine per molecule of pepsin   Of course

the solutions analyzed were reaction mixtures and in all probability not solutions of single compounds, nevertheless, the general effect of introduced iodine on the enzymatic activity of pepsin is indicated

Experimental Details

The procedure used in the experiment indicated in Fig 1, by the open circles o is as follows to 65 ml of a dialyzed 2 × crystallized Parke, Davis pepsin solution in 0.2 M pH 6 0 acetate buffer was added 4 ml of 1 N iodine in potassium iodide solution   The final concentration free iodine equals 0 06 N and protein nitrogen per ml equals 4 mg   The experiment was carried out at 5 C   At suitable intervals of time samples were removed   the free iodine destroyed by adding
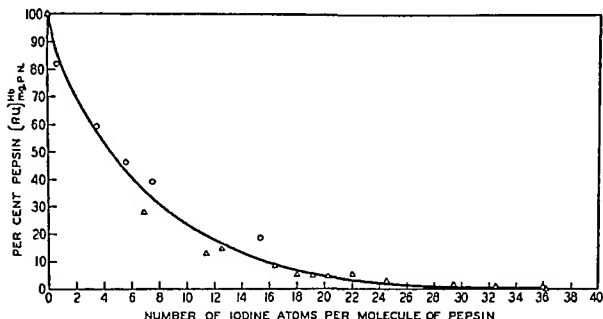

FIG 1   Effect of bound iodine on the specific activity of pepsin.

dropwise a solution of 10 per cent sodium bisulfite   The sample was then dialyzed in a rocking dialyzer (5) for 20 hours against M/2,000 pH 5 0 acetate buffer after which it was analyzed for protein nitrogen, proteolytic activity, and total iodine by methods outlined in the section of this paper devoted to Experimental methods

The procedure used in the experiment indicated in Fig 1 by the triangles, △ is as follows   to a solution of dialyzed 2 × crystallized Parke Davis pepsin containing 4 mg of protein nitrogen per ml was added an equal volume of 0 1 N iodine in potassium iodide solution   Aliquots were removed at intervals of time, treated, and analyzed as described above for the other part of this experiment

Oxidation and Substitution

Thus far in this paper only the "introduced" or "bound" iodine has been considered as the cause of the effects noted with regard to

enzymatic activity   Iodine may be acting in the present instance as an oxidizing agent as well as one of substitution   There exists, therefore, the possibility that the effect noted on the specific activity of pepsin is caused, not by the bound or substituted iodine, but by a simultaneous oxidation of some group in the protein molecule   The experiments shown in Table I were designed to determine to what extent oxidation by iodine takes place during the iodination of pepsin   When iodine acts as an oxidizing agent it is itself reduced from molecular iodine I^0 to iodide ion I^- The extent of oxidation may then be determined by estimating the production of iodide ion   It must be remembered, however, that in the iodine substitution reaction, as illustrated,

$$\text{Tyrosine} + 2\,I_2 \longrightarrow \text{diiodo-tyrosine} + 2\,H^+ + 2\,I^-$$
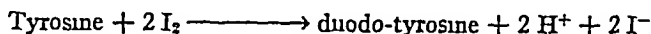
an amount of iodide ion is produced equal to that amount which substitutes   The iodide ion resulting from any oxidation by iodine must then be determined as that quantity of iodide ion over and above the iodide ion produced as a result of substitution   The iodide ion formation in the control experiment must, of course, be deducted

The writer was not able, unfortunately, to determine the iodide ion directly in the presence of iodinated pepsin and was forced to resort to estimating the extent of oxidation by an indirect method   If no oxidation takes place during iodination the organically bound iodine should equal one-half of the decrease in free iodine, as may be seen from the illustrated reaction   If oxidation does occur then the organic iodine will be less than one-half of the decrease in free iodine for some of the free iodine will be consumed in the oxidation.   Thus a comparison of the organic iodine determined and the organic iodine calculated as one-half of the decrease in free iodine will serve as a measure of the oxidation   Free iodine and therefore the loss in free iodine can be determined precisely by the usual iodimetric titrations   The figure for the organically bound iodine was obtained after dialyzing a sample of material free of all uncombined iodine   By making the nitrogen analysis before and after dialysis and iodine analysis after dialysis the organic iodine existing before dialysis may be calculated, assuming no loss of organically bound iodine during dialysis

Column (f) of Table I shows the difference between the organic

iodine determined and that calculated from the loss of free iodine
The results of these experiments are not as decisive as had been hoped
but the differences noted are small, indicating that little oxidation
occurs, and they seem to bear no relationship to the specific activity
of pepsin   It may be pointed out that using three different concen
trations of iodine in Nos 2, 4, and 10 of Table I and obtaining solu
tions with three quite widely different specific proteolytic activities,

TABLE I

Oxidation of Pepsin by Iodine at pH 5 6 and 5°C

No	Material	Original concentration of iodine	Time	P U Hb P N mg	Determined				Calculated	Difference
					Iodine/mL			Atoms I per mol pepsin	Decrease in free iodine / 2	(e) − (c)
					Total	Free	Organic			
					(a)	(b)	(c)	(d)	(e)	(f)
		normality	hrs		mg	mg	mg	mg	mg /ml	mg./ml
1	Pepsin	0 000	1	0 22						
2	Pepsin	0 005	1	0 15	0 63	0 00	0 23	5	0 32	0 09
3	Control (buffer)	0 005	1				0 65			
4	Pepsin	0 010	1	0 086	1 3	0 17	0 46	10	0 52	0 06
5	Control	0 010	1			1 2				
6	Pepsin	0 020	1	0 039	2 54	0 75	0 73	16	0 92	0 19
7	Control	0 020	1			2 6				
8	Pepsin	0 050	1	0 002	6 3	3 1	1 3	29	1 65	0 35
9	Control	0 050	1			6 4				
10	Pepsin	0 03	1	0 02	3 7	1 8	0 85	19	0 95	0 10
11	Pepsin	0 03	24	0 006	3 8	1 0	1 1	23	1 40	0 30

practically the same differences between calculated and determined
organic iodine may be seen in column (f)

Experimental Details

Materials—Dialyzed 2 × crystallized Parke, Davis pepsin in M/2 sodium
acetate pH 5 6, the solution contained 4 mg protein nitrogen per ml

Starting with 0 5 N iodine in 50 per cent ethyl alcohol, solutions of various
concentrations were made up by diluting this iodine solution with 50 per cent
alcohol   By dissolving the iodine in alcohol the presence of potassium iodide in
the solvent was eliminated

Procedure—With the solutions at 5°C, 15 ml of pepsin solution was mixed

with 15 ml of iodine solution and allowed to remain for 1 hour at 5°C Aliquots were then analyzed for protein nitrogen, activity by the hemoglobin method, total iodine, free iodine (direct titration), and the remainder of the solution treated with sodium bisulfite to destroy the free iodine remaining After treatment with sodium bisulfite these solutions were dialyzed for 20 hours at 5°C and then analyzed for total iodine and protein nitrogen A control on the pepsin activity was made by adding 15 ml of 50 per cent alcohol to the pepsin solution instead of the iodine solution A control on the oxidation of iodine was made for each concentration of iodine by adding 15 ml of iodine solution to 15 ml of M/2 pH 5 6 acetate buffer and then analyzing after 1 hour for total and free iodine

TABLE II

Oxidation during Iodination of Glycyl Tyrosine

No	pH	Time	Mg I/ml (determined)					Mg I/ml (calculated)			Extent of iodination
			Total	Free	Total free	Iodide	Organic	Organic	Iodide	Iodide	
			(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)
		hrs						$(c)_A - (d)_A$	$\dfrac{(c)_A - (d)_B}{2}$	$(d)_A - (d)_B$	*per cent*
1A	4 0	0 5	18 0	14 5	3 8	3 0	0 8	0 8	0 8	0 8	13
1B	—	0 5		16 5	2 3	2 2	0 0	0 1			
2A	—	72 0		8 3	10 5	6 4	4 0	4 1	3 9	3 9	65
2B	—	72 0		15 5	2 7	2 5	0 1	0 2			
3A	5 6	0 5	18 0	6 9	10 3	6 2	4 5	4 1	4 1	5 1	73
3B	—	0 5		15 7	2 2	1 1	0 2	1 1			
4A	—	72 0		2 3	13 6	7 7	4 9	5 9	4 9	5 2	84
4B	—	72 0		15 4	2 8	2 5	0 1	0 3			

In studying the iodination of glycyl tyrosine the iodide ion formed could be determined directly by a gravimetric method It may be worth noting that even in this case with direct methods of analysis and dealing with pure simple substances the results to be found in Table II are not perfectly constant There can be little doubt, however, that oxidation by iodine plays no part in this reaction

Experimental Details

Procedure —To 50 ml of glycyl tyrosine solution was added 25 ml of 1 6 M acetate buffer in one case pH 4 0 in another pH 5 6, followed by 25 ml of 0 5 N iodine in 50 per cent alcohol   As controls, equimolar solutions of glycyl glycine were substituted for glycyl tyrosine   In Table II the glycyl tyrosine is indicated as the 'A' experiments, while the control glycyl glycine experiments, are denoted by B"   The entire experiment was carried out at 5°C

At various intervals of time samples were removed and analyzed for the following properties (a) total iodine, (b) free iodine, (c) iodide ion plus organic iodine, (d) iodide ion, and (e) organic iodine

Analysis for the total iodine (a) was carried out by the procedure outlined in the section of this paper devoted to Experimental methods   The free iodine (b) was titrated with standardized thiosulfate   The iodide ion plus organic iodine (c) was determined by analyzing for total iodine after extracting the free iodine out of solution by shaking with several aliquots of carbon tetrachloride   The iodide ion (d) was estimated gravimetrically as the silver salt which is the only material precipitated in N/10 nitric acid solution   After removal of the iodide ion as silver iodide by filtration, the organic iodine was determined on the filtrate after first removing the excess silver ion by addition of an aliquot of N/10 sodium chloride Knowing the volumes used calculation back to the original aliquot was made

Effect of pH on Iodination of Pepsin and of Glycyl Tyrosine

In the studies on the acetylation of pepsin (1, 2) it was brought out that the phenolic hydroxyl group of tyrosine in pepsin and of pure glycyl tyrosine are both readily acetylated by ketene at pH 6 0 whereas at pH 4 0 acetylation is slow   Since iodine reacts with quite a different part of the tyrosine molecule than does the acetyl group it was of interest to see if the effect of pH was the same for iodination as had been observed for acetylation and the same for pepsin and glycyl tyrosine

Experimental Details

Materials —A dialyzed 2 × crystallized preparation of pepsin containing 6 3 mg of protein nitrogen per ml was used   With the exception of the buffer (citric acid) used to obtain pH 2 5 all the buffers were acetate buffers of molar concentration made up to give the indicated pH   The iodine was 0 15 normal in potassium iodide solution

Procedures —5 ml pepsin solution plus 5 ml 1 N buffer plus 5 ml 0 15 N iodine solution let stand at room temperature for 1 hour after which 1 ml was removed and titrated with standard thiosulfate to determine the free iodine remaining To the remainder of the solution was added dropwise 10 per cent sodium bisulfite solution until the free iodine was destroyed   The solution was then dialyzed for 20 hours against M/2,000 pH 5 0 acetate buffer at 5 C   Appropriate controls

were run at different pH in the absence of iodine to determine the extent of inactivation of pepsin, also in the absence of pepsin to determine the extent of oxidation of iodine by products other than pepsin

After dialysis the solutions were analyzed for protein nitrogen, activity by the hemoglobin method, and total iodine as described in the section devoted to Experimental methods

The results recorded in column (1) of Table II and Fig 2 show that, in general, during iodination of pepsin or of glycyl tyrosine a
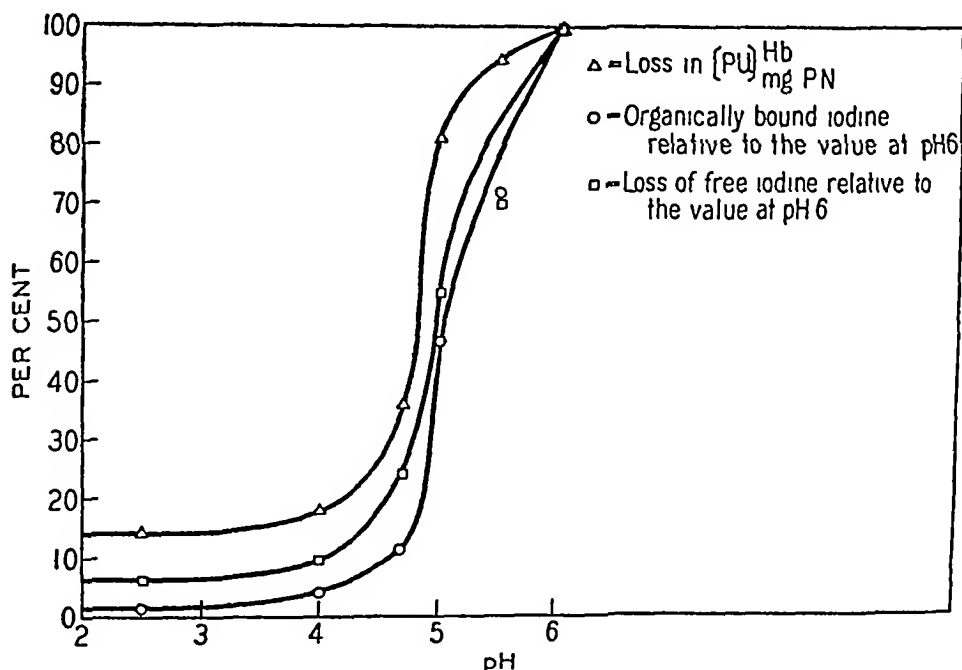
FIG 2 Effect of pH on the iodination of pepsin

change in pH of the medium alters the rate of iodination in much the same way as was noted for acetylation of these materials   According to the titration curve of glycyl tyrosine, determined by Greenstein (6) the carboxyl group is in the salt or zwitter ion condition at pH 5 0–7 0 but below pH 5 0 the carboxyl group ionizes as an acid

The results in Fig 2 bring out the fact that when free iodine is consumed iodine appears in the protein as bound iodine and the specific activity of the enzyme is depressed proportionally   If the decrease in specific activity were depressed as a result of oxidation by iodine, which cannot be completely excluded from the work of the

previous section of this paper it must then be supposed that the oxidation is affected by pH in an exactly parallel manner as is the introduction of iodine into the protein molecule   It should also be pointed out that since, as will be demonstrated later in this paper, at least 80 per cent of the iodine that is absorbed by the protein is attached to the tyrosine, to account for the depression of the pro teolytic activity on the hypothesis of iodination of some component of the protein other than tyrosine, one must again assume that the union of iodine with this hypothetical component is affected by pH in a manner parallel to that of tyrosine in pepsin

Isolation of Diiodo Tyrosine from Iodinated Pepsin

For the most part the work carried out in the isolation of diiodo tyrosine from iodinated pepsin is described in Table III   However, space would not permit inclusion of all the details and some points need explanation

Pepsin contains approximately 10 per cent tyrosine (7) which if completely iodinated (2 atoms of iodine per molecule of tyrosine) would result in a protein with 14 per cent iodine   The material used in this isolation experiment had 13 4 per cent iodine

It was found in preliminary experiments that pancreatic extract would digest the iodinated pepsin   Since this is a much less violent treatment than boiling in alkali it was decided to carry out the en zymatic digestion before treatment with alkali   It might be noted that the Bristol pancreatic extract used carries the digestion of the iodinated pepsin only one third as far as it does pepsin, as measured by the increase in Van Slyke amino nitrogen

Refluxing for 30 hours with barium hydroxide was necessary to complete hydrolysis even after the preliminary enzymatic hydrolysis By cooling to 5°C a large part of the barium hydroxide crystallized out and was separated by filtration

The butyl alcohol extraction proved to be an efficient and satis factory method of purification   The writer is indebted to Dr G L Foster for this suggestion

In No 11 of Table III the basic lead acetate precipitate was sus pended in about 75-100 ml of water and glacial acetic acid added The suspension was warmed to 50°C to aid in the solution of the

TABLE III

Isolation of Diiodo-Tyrosine from Iodinated Pepsin

Materials and procedures	Solution No	Volume	Total Nitrogen	Total Iodine	Original iodine	Phenol color iodine ratio*
		ml	mg	mg	per cent	
Dialyzed iodinated pepsin No 13 of 8/2/35 13 4 per cent iodine	1	515	2800	2400	100	0 57
Dialyzed Bristol pancreatic extract	2	5		27		
No 1 + No 2 + alkali to pH 9 0–10 0 at 37°C for 48 hrs 104 gm crystalline Ba(OH)$_2$ (making a 20 per cent solution) added and refluxed for 30 hrs , placed at 5°C for 24 hrs , residue filtered and washed with cold water, residue discarded, combined filtrates and washings	3	720	2200	2300	96	0 58
No 3 + 70 ml concentrated HCl (pH = approximately 0 7), shaken in separatory funnel seven times with 100 ml quantities of butyl alcohol (reagent grade)	4					
Acid-water solution of No 4 after last extraction with butyl alcohol	5	710		113	5	1 3
Butyl alcohol solutions of No 4 extracted two times with 150 ml quantities of 0 25 N NaOH and once with 0 1 N NaOH	6					
Butyl alcohol solution of No 6 after last extraction with alkali	7	380		38	1 6	
Alkali-water solution of No 6 after separation from the butyl alcohol	8	858	515	2400	100	0 46
No 8 + 20 ml of 50 per cent (by weight) PbAc$_2$ + 5 ml glacial acetic acid, left at 5°C for 20 hrs , filtered and residue washed on funnel with 0 1 N acetic acid	9					
Filtrates + washings of No 9	10	1370		2250	94	0 40
No 10 + concentrated NH$_4$OH to pH 8 0–9 0, let settle 24 hrs , filtered, residue washed by grinding in a mortar with 0 1 per cent NH$_4$OH and filtered Residue stirred with H$_2$O and 12 ml glacial acetic acid, warmed to 50°C stirred, filtered, residue washed with 0 1 N acetic acid	11†					
Residue No 11 (crystals) dissolved in dilute NH$_4$OH	12	300		780	33	0 25

* See section on Experimental methods for a discussion of these figures

† See text for discussion of the procedures from No 11 on

TABLE III—*Concluded*

Materials and procedures	Solution No	Volume	Total Nitrogen	Total Iodine	Original iodine	Phenol color Iodine ratio
		ml	*mg*	*mg*	*per cent*	
Solution No 11 + No 12 + NH₄OH to pH 8 0-9 0 allowed to stand 24 hrs filtered residue washed with water Residue suspended in 4–6 liters water at 40 C H₂S bubbled in till completely saturated Suspension stirred 6–10 hrs filtered residue re suspended in water H₂S bubbled in as before fil tered, filtrates combined evaporated under re duced pressure at 40 C	13	800		1980	82	0 29
Solution No 13 evaporated to 50 ml very poor crystallization solution very dark—became black on addition of NH₄OH to pH 8 0	14					
Precipitated with PbAc₂ at pH 8 0 and dissolved by acetic acid or by decomposition with H₂S repeated several times Fractional precipitation from di lute NH₄OH solution by addition of dilute acetic acid resulted in a tan colored crystalline product. A yield of 2 1 gm of dried material was obtained	15				53	0 25– 0 29
No 15 on recrystallization came down as bundles of needles at room temperature and as whet stones when the mother liquor was placed in the ice box From 50 per cent acetic acid the crystals were platelets or flat prisms After drying in vacuo at 100°C the crystals melted 204 C	16					

precipitate The precipitate was almost completely dissolved when a fine white precipitate began to form and became quite heavy A microscopic examination showed this precipitate to be very small crystals—too small to identify The crystals were filtered off, washed several times with N/10 acetic acid, and a sample of the residue analyzed It gave the color with the phenol reagent and iodine analysis expected of diiodo tyrosine and dissolved instantly in dilute ammonia The ammoniacal solution was combined with the mother liquor in solution No 11 in the hope of crystallizing the entire amount of iodine compound in the next step This, however, was not to be

the case   After the second precipitation with lead acetate at pH 8 0 and decomposition of the precipitate with hydrogen sulfide the solution was evaporated to 50 ml   Crystallization did not take place as had been hoped   The solution was cooled and even seeded with a few crystals of diiodo-tyrosine   A small precipitate appeared in 24 hours but the amount was insignificant   Precipitation as the lead salt and subsequent solution was repeated several times   Crystals or precipitates came out but they were always incomplete and dark brown or black in color   It soon became evident that there was some extraneous black material present in the solution which precipitated under the conditions for crystallization of diiodo-tyrosine and that its removal was difficult   That this black material prevented crystallization was proven to the writer's satisfaction when on adding acetic acid slowly to a dark ammoniacal solution of the unknown there appeared at first a black flocculent precipitate which settled leaving the supernatant liquor water clear   Almost immediately after the black material settled crystals formed in the supernatant liquor   In a few moments the container was nearly solid with a white crystalline product   Separation of the flocculent black material from the crystalline product was not so easily accomplished as might be supposed, for solution and precipitation conditions of both materials were almost identical   The separation was, therefore, for the most part fractional precipitation from dilute ammoniacal solutions by the addition of acetic acid followed by rapid filtration before crystallization occurred These procedures were very costly to the yield of crystalline product as may be seen from Table III   Although the solution No 13, just before the final fractionations were performed, contained 82 per cent of the original iodine and gave a color-iodine ratio nearly that of known diiodo-tyrosine, yet only 53 per cent of the original iodine was finally obtained in crystalline form, the difference being lost for reasons already stated   The black flocculent material contained no iodine and gave no color with the phenol reagent   No other analyses were made on it

The iodine product after recrystallization from dilute ammonia by the addition of a few drops of acetic acid and drying *in vacuo* had a melting point of 203°C uncorrected   Hoffmann-La Roche diiodo-tyrosine dried in a similar fashion melted at 204°C while a mixed

melting point of the two materials was 204°C   The phenol reagent color iodine ratio of the crystalline product was the same as that for the Hoffmann La Roche diiodo tyrosine

Titration Curve of Iodinated Pepsin

Although a large part of the iodine in iodinated pepsin was identified as diiodo tyrosine this in itself is not proof that the iodine was attached to the tyrosine in the intact iodinated pepsin   The vigorous treatment to which the protein was subjected in the process of isolation of diiodo tyrosine could conceivably cause the rearrangement or migration of iodine from some other position to the tyrosine

From the work of Neuberger (3) and others it is believed that when the tyrosine of proteins is iodinated the titration of the phenolic hydroxyl group of the tyrosine is shifted toward the acid region   If then tyrosine in pepsin is iodinated by the methods used, the titration curve of iodinated pepsin should be quite different from that of pepsin   It is to be expected that the curve of iodinated pepsin will show a rise in the curve between pH 8 0 and 10 0 instead of pH 10 0–12 0 as in pepsin   As may be seen in Fig 3 this is exactly as expected

Experimental Procedure

Materials—Iodinated pepsin   150 ml dialyzed 2 × crystallized Parke Davis pepsin, 14 mg protein nitrogen per ml plus 50 ml 4 x sodium acetate plus 300 ml water plus 500 ml N/10 iodine solution   Mixture left 72 hours at 37 C   Protein precipitated completely by acidification to pH 3 0, filtered and residue dissolved with the aid of sodium hydroxide   This solution was then dialyzed 36 hours against pH 6 0 acetate buffer at 5 C   The solution contained 4 0 mg protein nitrogen per ml and the protein was 16 per cent iodine

Pepsin   Dialyzed 2 × crystallized Parke Davis pepsin dialyzed 36 hours against pH 6 0 acetate buffer at 5 C   The solution contained 4 3 mg protein nitrogen per ml

Procedure—The hydrogen and calomel electrodes were standardized before and after each experiment with pH 4 0 acetate buffer prepared as recommended by Clark (8)   To 50 ml of protein solution was added 1 ml saturated potassium chloride and 1 drop of octyl alcohol and the hydrogen electrode saturated with hydrogen introduced   The solution was stirred throughout   0 05 ml quantities of 3 05 N alkali were run in through a capillary tube leading underneath the surface of the solution   A total of approximately 1 ml of the alkali was added   The E.M F was read from the bridge of the Leeds and Northrup potentiometer and the pH calculated   Correction was made for the quantity of alkali required to produce the same pH in a similar volume of distilled water

In Fig 3 the curve for iodinated pepsin does not meet the pepsin curve beyond pH 12 0 and there are several rises in the curve beyond pH 9 0   This was very disconcerting until it was remembered that hydrogenation of diiodo-tyrosine to give tyrosine is carried out under conditions (9) somewhat similar to those occurring during measurement of pH by the hydrogen electrode   That there was actual liberation of iodine from iodinated pepsin was shown by the fact that the nitrogen content of the titrated solution remained practically unchanged during dialysis whereas the iodine content was lower after
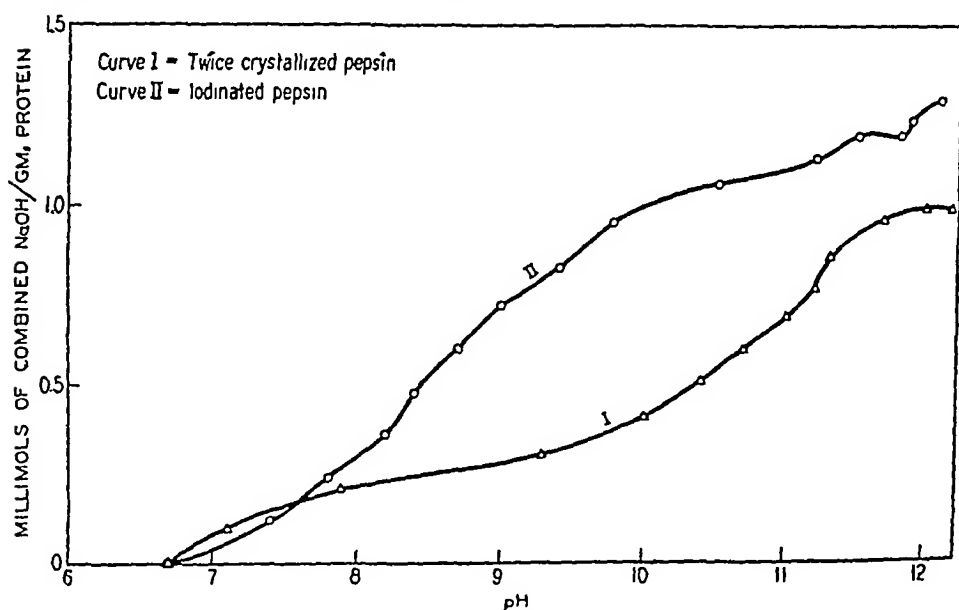
FIG 3   Titration curves of pepsin and iodinated pepsin

dialysis   The liberated iodine would be in the form of hydriodic acid and would therefore require additional alkali and make the total alkali per gram of protein appear to be greater in the case of iodinated pepsin than with pepsin

Neuberger (3) titrating zein and iodinated zein with a hydrogen electrode obtained curves which, although separated between pH 8 0 and 12 0 in the same way as herein demonstrated for pepsin and iodinated pepsin, nevertheless found that the two curves converged at pH 12 0   The time allowed for establishing equilibrium is probably responsible for the difference in the two cases   The writer

without considering the possibility of hydrogenation of the combined iodine consumed at least 2 hours in the measurements of each curve

It is interesting to note that from Fig 3 the amount of tyrosine iodinated in pepsin may be estimated   The difference between the pepsin curve I and the iodinated pepsin curve II at pH 10 0 is, roughly, 0 6 millimols of alkali per gram of pepsin or, in terms of tyrosine, is 11 per cent of the protein   The figure is remarkably close to the amount of tyrosine found (7) to exist in pepsin

DISCUSSION

The work on the acetylation of pepsin showed that the free amino groups of pepsin, which are probably the ϵ amino groups of lysine, could be acetylated without a detectable change in the enzymatic activity   On the other hand, acetylation of two or three tyrosine phenol groups caused a loss of 40–50 per cent of the enzymatic activ ity   The present work further indicates that alteration of the tyro sine of pepsin results in a change of the proteolytic activity   Whereas the acetyl group was attached to the phenolic hydroxyl group of the tyrosine in pepsin, the iodine substitutes in the benzenoid part of tyrosine, a quite different position, yet the effect of both additions is to reduce the peptic activity of the protein   It appears, therefore, that at least part of the tyrosine of pepsin is so arranged in the mole cule as to be essential for normal activity   The writer does not mean to imply that any part or all of the tyrosine is the hypothetical active group of the enzyme

Other proteins contain tyrosine and the other amino acids which have been found in pepsin and yet no other protein has the properties peculiar to pepsin   Since there is no evidence for the existence of a prosthetic group which, when separated from the rest of the molecule retains the proteolytic activity, the peptic action cannot then be attributed exclusively to any particular part of the protein molecule but must be due to the arrangement of its component parts   The work thus far reported by this writer indicates that in the arrangement of component parts the tyrosine plays an important rôle

Experimental Methods

Pepsin Activity —Measurement of the peptic activity was made by the hemo globin method of Anson and Mirsky (10)

Protein Nitrogen —This estimation was made as the difference between the total nitrogen and the non-protein nitrogen, the details being described in a previous paper (1)

pH —pH determinations were for the most part made colorimetrically using the indicators recommended by Clark (8)

Total Iodine —The total iodine estimations were made by a method which for the most part is the procedure of Kendall (11) with some additions or modifications In order that the changes may be scrutinized the entire scheme is included here

An aliquot of material to be analyzed containing 0 1–2 0 mg of iodine was introduced into a 6 × 6 cm nickel crucible   5 ml of saturated sodium hydroxide and approximately 5 gm of solid sodium hydroxide and a few alundum chips are added and the mixture heated on an electric hot plate, care being taken in the heating to prevent spattering of the boiling solution   When a crust begins to form on the hot alkali mixture the crucible is placed into a 7 × 7 cm nickel crucible over a Bunsen burner   A tripod is a convenient support for the larger crucible and the bottoms of the two crucibles should be separated by a few millimeters of fine sand   If the larger or outside crucible is then heated by the Bunsen burner until the lower third of it is red, the inner crucible will be about the right temperature   A gas (probably carbon dioxide) is given off from the alkali solution as small bubbles and upon their ceasing to appear a few small crystals of potassium nitrate are added and usually the evolution of gas commences again   Addition of a few crystals of potassium nitrate is repeated several times or until no gas appears on addition of the potassium nitrate   By means of crucible tongs the crucible is then lifted out and the melt poured onto a crucible cover   The crucible is held over the cover until that melt clinging to the crucible has solidified, this is usually only a moment   The solidified melt on the crucible cover is then put into a 500 ml wide mouth Erlenmeyer flask and the cover and crucible washed free of the melt, the washings all being introduced into the flask   The volume of the solution in the flask should be between 200–300 ml   When the melt is entirely dissolved 2 drops of 0 1 per cent methyl orange and 1 ml of 10 per cent sodium bisulfite are added to the flask and the solution titrated to a pink color with 85 per cent reagent grade phosphoric acid   A few drops in excess should be added   With the introduction of a few alundum chips the solution is then boiled on a hot plate for 2–3 minutes   The flask is removed from the hot plate and while still hot but not boiling 3 ml of saturated bromine water is added   The solution should remain yellow after mixing if enough bromine water has been added   3 ml is sufficient for up to 3 mg of iodine in the original sample   If the solution is not yellow more bromine water should be added after which the solution is boiled until colorless and then about 2 minutes longer   The flask is then set in running cold water and 4 drops of a 10 per cent solution of sodium salicylate are added to the solution   When cooled to room temperature a drop or two of 85 per cent phosphoric acid is added followed by 5 ml of a 10 per cent potassium iodide solution   It was found desirable to then add another drop or two of 85 per cent phosphoric acid to bring the full and rapid liberation of iodine   This solution was then immediately titrated with standard

0 01 N thiosulfate, each ml of which is equivalent to 0 21 mg of iodine in the original sample due to the nature of the reaction   3 ml of 0 5 per cent starch solution aids in determining the end point   The thiosulfate was prepared as recommended by Leland and Foster (12) in 0 005 N alkali   This solution was found to be perfectly stable for at least a month

Phenol Color Value —The phenol color value of solutions was obtained by use of the following procedure an amount of material which gave approximately the color produced by 0 15 mg of tyrosine was diluted with water to 19 ml   3 ml of 1 28 N sodium hydroxide was added followed by 3 ml of a 1 3 dilution of Folin's phenol reagent   The solution was allowed to stand 5 minutes for the color to develop after which it was compared in a colorimeter against 0 15 mg of tyrosine treated in a like manner

Since the reference or standard was tyrosine the values of all solutions analyzed were calculated in terms of tyrosine

The ratio found in Table III was obtained by dividing the color value of a solution in terms of milligrams of tyrosine by the milligrams of iodine of the same solution

SUMMARY

In the presence of iodine at pH 5 0–6 0 a solution of pepsin absorbs iodine and the specific proteolytic activity of the solution decreases The activity is less than 1 per cent of the original activity when the number of iodine atoms per mol of pepsin is 35–40

If the pH is 4 5 or less, iodine reacts very slowly and there is a correspondingly slower loss in activity   Glycyl tyrosine reacts with iodine in a manner similar to pepsin

Experiments were performed to determine the extent to which oxidation of pepsin by iodine occurs during iodination, and if such oxidation were responsible for the loss in enzymatic activity Although the results were not absolutely decisive, there seems to be no appreciable oxidation taking place during iodination and no relationship between the slight oxidation and loss in peptic activity

From a dialyzed preparation of completely iodinated pepsin which was inactive and contained 13 4 per cent bound iodine, 82 per cent of the iodine was obtained in a solution which analyzed as a solution of diiodo tyrosine   Because of the presence of a material which contained no iodine and prevented quantitative crystallization, only 53 per cent of the iodine containing substance could be crystallized This 53 per cent was, however, identified as diiodo tyrosine

The part of the titration curve which in pepsin and most proteins

represents the phenolic group of tyrosine was, in the curve for iodinated pepsin, shifted toward the acid region as expected

From these results, it appears that the loss in proteolytic activity of pepsin, when treated with iodine under the specified conditions, is due to the reaction of the iodine with the tyrosine in pepsin

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SEROLOGICAL REACTIONS OF AZOPROTEINS DERIVED FROM AROMATIC HYDROCARBONS AND DIARYL COMPOUNDS*

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The present study deals in part with the serological specificity of azoproteins derived from aromatic hydrocarbons other than benzene, namely the polynuclear compounds p aminodiphenyl, β naphthylamine and β anthramine, in addition, a number of substances containing two benzene rings—azoproteins from p aminodiphenyl ether, p amino diphenyl methane, p aminobenzophenone (and p aminophenylazo benzene)—, were examined because their reactions have been used to substantiate certain theoretical conclusions concerning serological specificity (Erlenmeyer and Berger (2))

Technique—Preparation of antigens To 4 millimols of the finely ground substances were added 10 cc N HCl and diazotization carried out at 0–5 C by addition of 4 cc N NaNO₂ With β naphthylamine and β anthramine diazotization was carried out in 5 N acid and the excess acid nearly neutralized by addition of a requisite amount of NaOH to the serum before coupling The solutions were filtered added to a cold mixture of 70 cc normal serum and half that volume of N Na₂CO₃, coupled for 10 minutes at a reaction alkaline to phenolphthalein and precipitated if not already out of solution with 10 per cent HCl After washing three times with saline and passing through gauze to break up lumps the azo proteins were dissolved in saline solution by adding at once enough N NaOH to bring the solution to N/20, (and in some cases a very small quantity of alcohol), the solution neutralized within a few minutes by cautious addition of N/10 HCl, and diluted with saline to a concentration of approximately 1 0 per cent of protein With some antigens, to have clear solutions for the tests the stock solution was clarified by a drop of N NaOH, which was subsequently diluted with saline to the proper concentration

Immunizations—Rabbits were given intravenous courses of 6 daily injections of

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20 mg each on alternate weeks and bled 1 week after the last injection   The antigens were prepared from horse serum   In several cases difficulty was encountered in obtaining sufficiently potent antisera, particularly with β-anthramine, for which only one serum was obtained

Tests—Antigens were prepared from chicken serum, except as specified Antigen dilutions are given in terms of a 5 per cent solution   The intensity of reactions is indicated as follows 0, f tr (faint trace), tr (trace), $\pm$, $\div$, $+$, $+\pm$, etc

The reactions of antisera for azoproteins from β-anthramine, p-aminodiphenyl, p-aminodiphenyl methane and aniline were tested with the homologous antigens and others made from β-naphthylamine and p-toluidine   The results are given in Table I   It will be observed that the azoproteins from aniline and toluidine were rather well differentiated from the polycyclic compounds aminodiphenyl methane, aminodiphenyl, β-naphthylamine, and β-anthramine   Naphthylamine antigen reacted with antiserum to β-anthramine as well as with the antisera to the other two polycyclic compounds, which appeared to be quite similar when tested with a diphenyl serum   In general, differences were greater with increasing disparity in structure, thus only a slight cross reaction was observed between aniline and β-anthramine azoproteins

The reactions of the other antisera and antigens are summarized in Table II   According to Erlenmeyer and Berger antisera to aminodiphenyl methane and aminodiphenyl ether antigens were identical in their serological reactions, due to the similarity of CH_2 and O in electronic structure, whereas the antigen from aminobenzophenone containing, instead, the group $C=O$, not related to CH_2 and O in this respect, would react with "diphenyl methane antiserum" not at all, and but slightly with antiserum to diphenyl ether antigen   This, along with other of their observations, was used as an argument for the hypothesis that compounds isosteric in the sense of Grimm (3) (which replace each other in crystals) are serologically equivalent

Not quite in accordance with this our results showed that when the test antigens were prepared with another protein (chicken serum) than that used for immunization several diphenyl methane sera, *e g* serum I, were found to show a definite difference between the diphenyl methane and diphenyl ether antigens   Some diphenyl ether and diphenyl methane antisera gave no or weak reactions with benzo-

phenone antigen but others gave quite distinct precipitation with this azoprotein, and the benzophenone antisera reacted with diphenyl ether and diphenyl methane antigens although markedly less than with the homologous one

While, as mentioned above, diphenyl methane and diphenyl ether sera were found which reacted strongly with the two corresponding antigens and weakly or not at all with benzophenone antigen after 1 hour, in later readings definite reactions were mostly seen   Antisera to p aminodiphenyl gave distinct reactions of similar strength with all three compounds, contrary to the assumption that the $-O-$, $-CH_2-$, and $-CO-$ groups are decisive for the specificity of these compounds Thus it would appear that the serological difference between diphenyl ether and diphenyl methane is definitely, but not very much, less than that between these two compounds and benzophenone

Since Erlenmeyer and Berger (2) used antigens prepared from horse serum both for immunization and testing it was thought that the discrepancies might be accounted for by this technique   Accordingly, p-aminodiphenyl ether and p aminodiphenyl methane antisera were tested against the homologous antigens and that of p aminobenzophenone all prepared with horse serum, with the result shown in Table III   Now the difference between diphenyl ether and diphenyl methane was obliterated for the most part as in Erlenmeyer and Berger's experiments and some of these sera failed to react with p aminobenzophenone antigen with the small amount of antiserum used, although others gave distinct reactions However in experiments where the protein used is the same in the test as in the immunizing antigens the reactions will partially depend on this common component [1]   With this in mind tests were made with immune sera to substances that would not be expected to react with these compounds to any considerable extent and here again the benzophenone antigen was found to be less reactive with some sera (Table IV), a result which cannot be explained on the basis of the difference between the CO O and CH_2

On account of an error due to a mislabelled commercial preparation (since mentioned by Landsteiner (5)) another of the cases advanced by Erlenmeyer and Berger to support their theory namely the reactions of two aminobromosulfonic acids can no longer serve as an example   Here it appeared that the interchange of the substituents NH_2 and Br did not affect their specificity   However, the two preparations considered to be 4-bromoaniline-2 sulfonic acid and 4-bromoaniline 3 sulfonic acid were later found to be the same substance namely 4-bromoaniline 2 sulfonic acid   On testing azoproteins from preparations of the two acids

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[1] A criticism concerning this point was made al o by Heidelberger (4)

named, made in this laboratory, they proved to be serologically different (Table V), moreover a clear distinction between the two acids was obtained in inhibition reactions as shown in Table VI   Thus from the inhibition reactions NH_2 and Br or Cl appear to be serologically not equivalent, the evidence from precipitation tests with azoproteins does not, of course, serve to compare these substituents, since NH_2 groups have been changed to $N = N$

Returning to experiments on the specificity of hydrocarbons, it may be said that, so far, they have been made on a small scale only   They seem to indicate that considerable differences exist, especially between benzene and its derivatives and some of the polynuclear rings   The continuation of these experiments would be desirable, especially from the point of view of whether or not the specificities which are recorded in Table I indicate that the carbon rings as such take part in the union with antibodies and are the directly determining factor in the reactions

SUMMARY

Serological reactions of azoproteins from p-aminodiphenyl, β-naphthylamine, β-anthramine and some compounds containing two benzene rings (aminodiphenyl methane etc) have been described, the last mentioned substances having been the subject of theoretical discussion on serological specificity

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TABLE I

2 drops of the immune sera from aniline ammodiphenyl and ammodiphenyl methane and 4 drops of the one from anthramine were used Dilution of chicken antigen was 1 100 Readings were taken after 1 hour at room temperature —first line—and after standing overnight in the ice box—second line Combined table
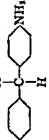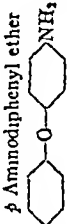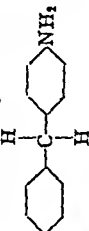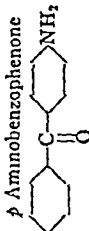
Immune sera	Antigens from					
	Aniline NH₂	p-Toluidine H₃C NH₂	p-Ammodiphenyl methane H₂N⟨⟩CH₂⟨⟩NH₂	p-Amined phenyl NH₂	β-Naphthyl amine NH₂	β Anthramine NH₂
Aniline	+++ / ++++	+++ / +++	f tr / ±	0 / ±	0 / ±	0 / tr
p-Ammodiphenyl methane	0 / tr	0 / ±	++ / +++	0 / ++	± / ++	0 / ±
p-Ammodiphenyl I	0 / f tr	f tr / ±	++ / ++±	++ / +++	++± / ++	tr / ++±
p-Ammodiphenyl II	± / +±	± / ++	+± / ++	+± / ++±	++ / ++	
β Anthramine	0 / 0	0 / 0	0 / 0	0 / tr	± / ++	+ / ++

TABLE II

2 drops of immune serum used in all cases Dilution of chicken antigen 1 500 Readings were taken after 1 hour at room temperature—first line—and after standing overnight in the ice box—second line Combined table
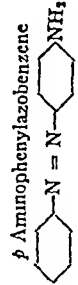
Immune sera	Antigens from chicken serum and				
	p Aminodiphenyl	p Aminodiphenyl ether	p Aminodiphenyl methane	p Aminobenzophenone	p Aminophenylazobenzene
p Aminodiphenyl	+ / ++	+ / ++	+ / ++	+ / ++	+ / ++
p Aminodiphenyl ether I	0 / +	++ / ++	++ / ++	++ / ++	tr / +
p Aminodiphenyl ether II	f tr / 0	++ / +++	+ / ++	tr / ++	++ / +
p Aminodiphenyl methane I	0 / 0	0 / ±	++ / +++	0 / tr	0 / tr
p Aminodiphenyl methane II	0 / +	++ / ++	± / ++	++ / ++	f tr / tr
p Aminobenzophenone	0 / f tr	tr / +	± / ++	++ / +++	0 / tr
p Aminophenylazobenzene	tr / +	++ / ±	± / ++	± / ++	++ / ++

TABLE III

Horse serum used in preparation of antigens   Antigen dilutions 1 500
1 drop of immune serum used in each case   First reading taken after 30 minutes
at room temperature (first line), second reading made after 1 hour at room
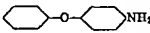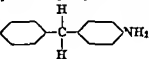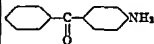temperature with sera I and after standing overnight in the ice box with sera II
(second line)

Immune sera	No	Antigens from		
		p-Aminodiphenyl ether	*p* Aminodiphenyl methane	*p*-Aminobenzophenone
		⬡—O—⬡—NH₂	⬡—CH₂—⬡—NH₂	⬡—CO—⬡—NH₂
p Aminodi phenyl ether	I	+ ++	+ ++	+ ++
	II	+± +++	± +++	0 tr
p Aminodi phenyl methane	I	+ ++	+ +±	± +±
	II	+± +++	+± +++	0 0

TABLE IV

Antigen (horse) dilutions 1 500   2 drops of immune serum used except in the case of anthranilic acid antiserum of which 1 drop was used   Readings were taken after 1 hour at room temperature—first line—and after standing overnight in the ice box—second line
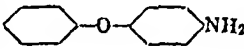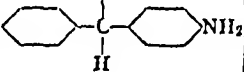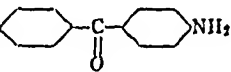
Immune sera	Antigens prepared from horse serum and		
	p Aminodiphenyl ether	p Aminodiphenyl methane	p Aminobenzophenone
Normal horse serum	0 tr	0 f tr	0 0
Aniline	+ +±	+± ++	0 ±
Aminodiphenyl	++ ++±	++ ++±	0 tr
Aminophenylazo benzene	++ ++±	+ ++	± +
Anthranilic acid	++ ++	+± +±	f tr ±

TABLE V

Antigen (chicken) dilutions 1 500   2 drops of immune serum used in each case   Readings taken after 1 hour at room temperature
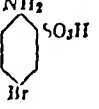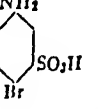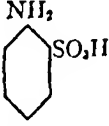
Immune sera	Antigens from			
	NH_2 SO_3H	NH_2 SO_3H	NH_2 SO_3H Br	NH_2 SO_3H Br
NH_2 SO_3H Br	+±	0	++	0
NH_2 SO_3H Br	+	+±	f tr	++±

TABLE VI

To 0 05 cc of each solution containing 1 millimol in 10 cc was added 0 2 cc of a 1 500 dilution of the homologous chicken antigen, followed by 2 drops of immune serum Readings were taken after 15 minutes—first line—and after standing overnight in the ice box—second line

Immune sera	Substances tested for inhibition						Control
	1 (NH₂, SO H, Cl)	2 (NH₂, SO H, Cl)	3 (NH₂, SO₃H, Br)	4 (NH₂, SO₃H, Br)	5 (NH₂, SO₃H, CH₃)	6 (NH₂, SO₃H, CH₃)	
NH₂, SO₃H, Br	0	tr	0	f tr	0	±	+±
	f tr	++±	0	+±	±	++	++±
NH₂, SO₃H, Br	tr	0	tr	0	±	f tr	++±
	++±	f tr	+±	0	++	++	++++

CRITICAL ILLUMINATION AND CRITICAL FREQUENCY FOR RESPONSE TO FLICKERED LIGHT, IN DRAGONFLY LARVAE

BY W J CROZIER ERNST WOLF AND GERTRUD ZERRAHN WOLF

(From the Biological Laboratories, Harvard University, Cambridge)

I

The interpretation and the theoretical implications of measurements of reaction to flickered light have been given preliminary con sideration in the case of data obtained with the honey bee and with the sunfish *Lepomis* (Crozier (1935–36), Crozier, Wolf, and Zerrahn Wolf (1936–37)) For a variety of reasons it was desirable to extend the observational foundation for the proposed development Curves expressing critical illumination as a function of flicker frequency (Wolf (1933–34), Wolf and Zerrahn Wolf (1935–36 b)) have been obtained from homogeneous data for *Apis* and for *Lepomis*, and for critical frequency as a function of illumination with *Lepomis* (Crozier, Wolf, and Zerrahn Wolf (1936–37)) The reciprocal curves for the same organism, *Lepomis*, are not identical their differences have been in certain essential respects predicted (*cf* Crozier (1935–36), Crozier, Wolf, and Zerrahn Wolf (1936–37)) The meaning of these differ ences is of prime significance for the attempt to utilize such measurements for a theory of the mechanism of excitation, one important aspect of their meaning concerns the propriety of utilizing *averages* of measurements of reactiveness, in the way which has become almost axiomatic but which seems to be incorrect We have proposed to test further the reality of these differences, and the capacity to pre dict their interrelations, by means of additional experiments with another animal For this purpose we chose the larvae of a dragon fly, *Anax junius* In the present paper we describe the results se cured when the relationship between flicker frequency and illumina tion for threshold response is concurrently measured in each of the

two ways conveniently and necessarily open to examination, namely by (1) determining mean critical flicker frequency (F_m) as a function of illumination (I), and (2) mean critical illumination (I_m) as a function of frequency of flicker (F) The two curves differ in predictable ways, entirely consistent with the results we have already analyzed in the case of *Lepomis*, moreover, they are also concordant with the interpretation to which the data on *Apis* have been submitted It has seemed to us important to carry through this experiment with two organisms (*Apis, Anax*) in which the eyes are of the same general sort, and which presumably possess but a single type of visual cell, the duplex composition of the typical vertebrate retina presents a special problem, since the respective contributions of rods and cones are to be separated in the analysis of the result when the whole eye is open to excitation A following paper deals with the use of these findings in devising an interpretive theory of response to visual excitation as it involves the discrimination of intensities

The predictive requirements of the situation are derived from the view that threshold response to flickered light is a matter of intensity discrimination (Crozier (1935–36)), and involves the statistical comparison of groups of effects (1) The curve for F_m as a function of I should lie above that for I_m as a function of F, (2) the shape of the two curves should not be the same, and the discrepancy between them (measured by $F_m - F$) should pass through a maximum as I increases, at the inflection of the $F_m - \log I$ curve, (3) the variation of I ($= \sigma_I$) should be directly proportional to I_m, when the critical illuminations are measured at fixed flicker frequencies, (4) the variation of F, when F_m is determined at fixed illuminations, should pass through a distinct maximum near the inflection point of the $F - \log I$ curve

We have already considered some of the evidence proving that the variation of the measured quantities in such experiments is, under suitable conditions, a property of the system of which the performance is under examination as an index of excitability (Crozier (1935), (1935–36), Crozier, Wolf, and Zerrahn-Wolf (1936–37)) This being the case, serious obstacles confront any attempt to pass from a series of homogeneous averages, as of intensity to produce a certain effect, to a mechanism for the basis of the effect One conclusion emerging

from this is, that the mean increment of intensity ΔI just recognizable as greater than a given intensity (I_1) is primarily a statistical quan tity, a property of a parameter of a frequency distribution, which possesses certain novel characteristics   In a succeeding paper it is shown that the practical recognition of these characteristics appar ently brings about a unifying interpretation of all available relevant data pertaining to tests in which phenomena of intensity discrimina tion are the basis of the measurements

The investigation of these matters is confronted by certain intrinsic difficulties which it is believed can be circumvented by a procedure of the general sort which we have developed   It is obvious that in any quantitative investigation of the reactive capacity of a given organism one is concerned with sources of variation arising in (1) the nature and use of the instrumental aids to measurement their ultimate precision and structural inadequacies and (2) the intrinsic variability of the reacting organism   The procedural scheme we have adopted based largely upon experience (cf Crozier (1929) (1935)), is in outline this to obtain at each of a number of magnitudes of an independent variable the same number of estimations (of the same or as nearly as possible the same inherent precision so far as the adjunctive measuring devices are concerned) of the reactive capacities of the same individuals   Means of these measurements for these individuals are then aver aged, thus minimizing the instrumental errors '' and the dispersion of the indi vidual means gives an estimate of the individual variability when this index of dispersion is considered as a function of the independent variable a measure is obtained of the organism s capacity to vary its performance as a function of the fixed conditions (Crozier (1929) (1935))   The adequacy of this is attested chiefly by (1) the consistent account thus empirically secured of the capacity to vary per formance—and the incidentally demonstrated absurdity of the idea that organic variability is complex and indeterminate —and (2) by the demonstration which we shall illustrate that a given group of individuals of one type enables one to sample the capacity for variance which any single individual of these can exhibit   The experimental difficulty here is, that to obtain adequate data for *direct* proof of (2) would involve tests over a length of time which is in general prohibitive and impracticable partly because the single tested individual might be expected to change in a significant way during the tests   The proof is thus neces sarily in part statistical but appears to be reasonable and adequate   We do not give any extensive account of the application to these data of the method of analysis of variance (Fisher and MacKenzie (1923)) largely because the consist ent use of the *same* individuals introduces an unknown restriction upon degrees of freedom in comparing the different tables of data, the limitation here is properly termed organic ''and is not of the sort contemplated in routine statistical practice (cf Crozier and Pincus (1935-36))   It should be said however that the general cor

relation method (variance analysis) has been applied to these data, without yielding any additional information or guidance beyond that given by the methods here used

II

The 12 individuals employed for these tests were selected from a lot secured in November, 1935, they were numbered and kept in separate aquaria   They were fed at regular intervals   2 hours before the start of an experiment they were transferred to cylindrical jars, 10 cm in diameter and holding 220 cc of water, and put into darkness   The temperature was $22° \pm 0\ 5°$, during an experimental run the temperature was $21\ 5° \pm 0\ 4°$ A jar was placed on the glass-topped table, surrounded by a striped screen, in the apparatus illustrated in our preceding papers (Wolf and Zerrahn-Wolf (1935–36b), Crozier, Wolf, and Zerrahn-Wolf (1936–37))   Screens with 5, 10, 20, and 40 opaque vertical stripes (alternating with transparent stripes of equal widths) were used to produce various controlled flicker frequencies (F) The method of control and measurement of F has been described in the preceding paper, the method of controlling and estimating the intensity of illumination was also the same as that previously used, in the present experiments light sources of 100 and 1000 watts were used, without neutral filters, no properties of the data are correlated with a difference in the primary source of light

After being dark adapted, and subsequent to an interval of adjustment to allow for any disturbance due to handling, the striped cylinder is set into motion at a speed of rotation giving a certain fixed flicker frequency (F) The light is then turned on, with the diaphragm closed   The diaphragm is then slowly opened (by a gear transmission) until the first response to the moving stripes is apparent The diaphragm opening is then noted, by means of calibration charts the illumination I is obtained from this reading   At each flicker frequency, with each animal, three such determinations are made in succession   From these three a mean intensity (I_1) is gotten for each individual, at each F The average of the twelve values of I_1 gives the mean intensity I_m This procedure is used (Crozier, Wolf, and Zerrahn-Wolf (1936–37), and page 367) because it minimizes errors of estimation of I due to the technique and permits an examination of the intrinsic variability of response   With the diaphragms set to give fixed illumination $= I_m$, the larvae are again examined to determine the critical frequency of flicker for reaction The striped cylinder is set into rotation at a speed giving a flicker frequency much higher than the critical, the light is then turned on, the larva remains quiet, the flicker frequency is then reduced slowly, until a response is obtained, at this instant the voltmeter connected with the magneto actuated by the driving shaft is read by another observer, and a shutter interrupts the light while the flicker frequency is again set at a high level   A second and a third reading are then taken in the same way   These are averaged for each individual, as in the case of the intensity readings

The reaction taken to signify excitation by the flickered light consists typically in a swimming movement in the direction of the moving stripes   In certain cases the larva does not swim but lifts the head, turns toward the stripes, and after a few

seconds extends the labium toward the stripes ("catching reaction") This response occurs at the same levels of F and I as does the swimming movement The readings used, however, are based upon the occurrence of the initial swimming movement It is very important that the larva should be quiet before a new reading is attempted any movement influences the apparent value of the critical I or F

<div align="center">III</div>

The observations have been treated in the same manner as in our experiments with *Lepomis* (Crozier, Wolf, and Zerrahn Wolf (1936–37)) The three diaphragm readings at each fixed F are averaged to obtain from the calibration curves the critical intensity for response in each individual The intensity figures for the twelve individuals are then averaged to give I_m, and the P E of the set of twelve is computed This is done to reduce the adventitious errors of making and reading the intensity settings The mean P E of the separate deviations for the twelve individuals from their respective means is quite small, and amounts to 1 3 to 1 6 per cent of the mean diaphragm reading, this is precisely the mean error of a setting of intensity in the original determination of the calibration curves of the apparatus, and is independent of the intensity and of the individual animal The individual means differ among themselves to a much greater extent and their dispersions exhibit an orderly relation to the in tensity The use of mean standard deviations of individual de partures from individual means of performance, for the analysis of data in genetically homogeneous material, has been illustrated in another case (Crozier and Pincus (1935–36)) where the number of measurements with each individual was larger and each datum was, in the nature of the measurement itself, an "internal average" just as in the case of response to flickering light

Any one individual does vary from time to time in reactiveness, and it is a major aim to investigate this variation because its properties give a clue to the real meaning of the average critical intensity for the threshold response (movement), but this variation, as will be proved, is at a much slower pace (as a rule) than could be reflected in measurements made in rapid succession It is quite clear, however, that repeated sets of determinations of I_m at any fixed F, with one individual, would show over a long time precisely the sort of variation

given by the different individuals used at one time   The behavior of the dragonfly larvae differs from that of the fishes used in our previous experiment in one significant detail—the fish is predominantly *quiet* up to the moment of definitive reaction, whereas the *Anax* larvae are often in slight motion,—perhaps crawling, perhaps propelled by the rectal pump   This might be expected to make the readings more variable, since the occasional state of slight motion, or incipient movement could modify the apparent threshold for excitation   There is no evidence in the data, however, that this is a recognizable factor

It is of some importance that there is no relationship between rank-order position as to relative sensitivity or mean sensitivity and chronological sequence of the tests over the period involved in the experiment   The time-order of intensities used in securing the data of Table V was essentially random   It is given here

Order of sequence in time	1 2	3	4	5	6	7	8 9	10	11 12 13 14 15 16 17 18 19 20
Flicker frequency tested	10 5	25	30	35	20	16	12 5	8 6	72 3 33 2 45 55 60 61 58 50 40 25

It is more important that the readings show no progressive change during the three determinations at each setting   This rules out any disturbance due to adaptation during a test   As the data in Table V show, altering the gear ratio or the stripe system on the rotated cylinder has no influence upon I_m, F_m, or the dispersions of I_1 or of F_1

It might be thought better to employ the dispersions of the sets of 36 measurements   We have preferred to utilize the method of "internal averaging" because it conveniently minimizes the influence of adventitious errors of measurement   The values of I_m, however, and of P E $_{I_1}$, computed on this basis, have exactly the same general properties as those given in Table V

The data on critical flicker frequency have been treated in a precisely analogous way   Both sets of data demonstrate that there

has been no change of mean sensitivity during the course of the series
of measurements, there is thus no evidence for progressive change in
the animals, nor in the observer's use of his criteria for the occurrence
of response   An interesting subsidiary point has to do with the
fact that on two occasions a larva moulted several hours before a
test was made, there was no change in its sensitivity before or after
this occurrence

<div align="center">TABLE I</div>

Average rank-order positions (R_I, R_P) of relative sensitivity in 20 sets of
determinations of mean critical intensity and of mean critical flicker frequency
for response for 12 individual larvae of *Anax*   The rank-order numbers in any
one set of measurements (see text) are assigned in the order of *increasing* intensi
ties (*I*) required to evoke response, and in the order of *decreasing* flicker fre
quency (*F*)

Animal No	Mean relative sensitivity	
	R_I	R_F
1	7 42	6 41
2	7 17	7 23
3	8 07	7 35
4	6 92	5 95
5	8 17	7 45
6	4 10	5 96
7	4 72	5 68
8	6 36	7 48
9	6 51	6 55
10	7 00	5 75
11	5 15	4 85
12	6 85	6 86
Mean	6 53 ± 0 781	6 46 ± 0 612

In order to show the necessity for considering the measurements of
I separately for each individual we may examine the *relative* sensi
tivities of the 12 individuals in the various sets of measurements
This is best done by means of a rank-order scale   In any one set of
tests the individual reacting with the lowest value of I_e is numbered
1, the next 2 and so on   From the group of 20 sets of tests a mean
rank-order position is then computed for each individual   In the
same way, from the determinations of *F* at fixed values of *I* rank

order numbers are assigned (in the sequence of *decreasing* magnitudes of F_c) and mean rank-order numbers again obtained   These are listed in Table I   The *mean* rank-order index of relative sensitivity is distributed in a completely random manner, for R_I and for R_F, the extreme deviates are 3 × the P E of the dispersion, and the ex-

TABLE II

Correlation of mean rank-order positions from intensity determinations (R_I) with mean rank-order positions from flicker frequency determinations (R_F) for 12 individual larvae of *Anax*

		Mean R_F			
		4 0–4 9	5 0–5 9	6 0–6 9	7 0–7 9
Mean R_I	4 0–4 9		2		
	5 0–5 9	1			
	6 0–6 9		1	2	1
	7 0–7 9		1	1	1
	8 0–8 9				2

TABLE III

Correlation between rank-order positions from intensity determinations (R_I) and rank-order positions from F_m determinations (R_F) made shortly afterward (same afternoon)   (See Fig 1)

		R_F						
		1 5	3 5	5 5	7 5	9 5	11 5	Mean R_F
R_I	1 5	6 50	10 00	6 00	9 25	4 75	5 25	5 08
	3 5	10 00	12 50	8 75	7 00	3 75	—	4 64
	5 5	10 00	4 50	12 25	7 00	5 25	4 00	5 76
	7 5	6 50	5 50	9 50	6 00	8 00	7 50	6 71
	9 5	6 00	5 00	7 50	7 50	10 50	8 50	7 14
	11 5	4 00	5 00	2 50	3 50	11 50	15 00	8 31

treme differences are $= 2\ 3$ P E $_{Diff}$ in each case   However, mean R_I shows positive correlation with mean R_F (Table II)   But this does *not* signify that certain individuals are consistently more sensitive than others   Determinations of I_1 and of the associated F_1 were made at one intensity on each day (in one afternoon)   When the relation of I_1 to F_1 rank-order positions is studied in the data of the

same day, the correlation is marked (Table III), and the regression of mean R_{F_1} upon R_{I_1} is definite and significant (Fig 1) The correlation completely disappears when the intensity sensitivity order on one day is related to the intensity sensitivity order on the *next* day,

TABLE IV

Exhibiting absence of correlation between rank-order position for intensity determinations (R_I) on one day and rank-order position from flicker frequency determinations (R_F) made on the *next* day

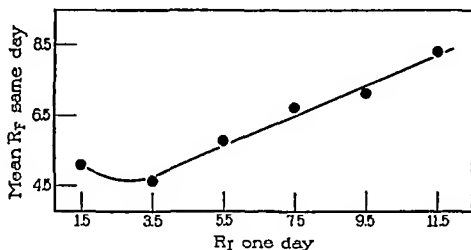|		R_F			
		2	5	8	11
	2	12	15 50	14 50	16
R_I	5	18 50	10 75	14 75	11 50
	8	13	11 50	19 50	14 50
	11	16 50	15 75	9 25	13 50

FIG 1 Mean rank-order number from determinations of F_1 associated with rank-order classes of the same individuals based on determinations of I_1 *in tests made within 2 hours on the same day* See text

or to the flicker frequency sensitivity order on the next day (Table IV) The same general relationship appears in two other series of measurements made in a subsequent experiment This means that the relative sensitivities of the 12 individuals are adequately indicated by their respective averages of I_1 and F_1, and that the individual sensitivities tend to be maintained for a period of several hours, but not for 24 hours The form of the regression of F_1 rank-order position

upon I_1 position shows that the relatively most sensitive condition of an individual is maintained longer than its least sensitive state This can be pictured as due to fluctuation of an individual's relative sensitivity with time, in which the cycle of changing sensitivity is not sinusoidal but asymmetrical and rather flat-topped, the rising phase being more abrupt, and with briefer depressions to minimum sensitivity, thus the more sensitive individuals after a given interval which is not too long are found to preserve more distinctly their rank-order positions than do the less sensitive It is easily seen that if the interval between them is not too long two determinations of sensitivity (R) with a single individual will then be related, on the average, in such a way that for a high value of sensitivity in the first measurement (R') there will be found a slightly lower mean value in the second (R''), for the lowest value in the first determination a distinctly higher value in the second, but that for an intermediate value (on the low R side) R'' must pass through a minimum

The change in sensitivity with time is completely out of phase in the several individuals—as proved by the random distributions of the mean rank-order positions Therefore it is not due to the time of day or to other events which might control the individuals as a group The absolute amplitude of the cycle of changes in sensitivity which must be assumed depends upon the intensity of the light, since the ordinate span of units of sensitivity (1 to 12) has the same kind of meaning as P E $_{I_1}$ and is proportional thereto One is consequently justified in supposing that there must exist a connection between the fluctuation in sensitivity (on the rank-position scale) and the variation in I_1 for threshold response to flicker A line of reasoning has already been suggested according to which response to flickered light depends upon a discrimination between E_z, the effect of a light flash, and $E_z - \Delta E_z$, the persisting effect in the dark interval (Crozier (1935–36)) In the nature of the case both E_x and $(E_x - \Delta E_x)$ are averages, and are functions of I_z, with equal dark and light sectors I_z may be assumed to be about 0 5 I, or at least $= nI$, where I is the acting intensity of the light flash From the directly ascertained data of intensity discrimination ΔI is proportional to P E $_{\Delta I}$, and so to P E $_{I_{(z)}}$ (Crozier (1935–36)) Hence the fineness of the discrimination which can be made to meet a fixed criterion (threshold response to

flicker) is related to the frequency distribution of the effects produced by the acting intensity, and particularly to the dispersion of these effects   Consequently $P E_{I_1}$ calculated from the 12 values of I_1 at each intensity measures the dispersion of "effects" produced by I_m in the states of the reacting organic system open to detection or measurement under the conditions of the present tests   The coherence of the data shows that using these 12 individuals at one time merely amounts to a convenient way of examining 12 randomly distributed reactive states of this system at about the same moment   The behavior of $P E_I$, for example, as a function of I_m, is precisely
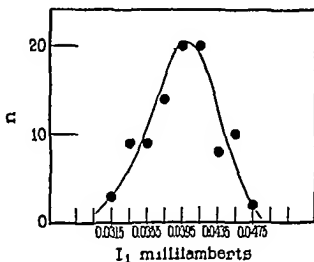

FIG 2 Frequency distribution of 95 determinations of I_1, the critical illumination for response to flickered light with flicker frequency fixed at $F = 25$ per second The distribution is not skewed, shows no preponderance of low intensity readings

the same as that obtained in cases where single measurements are made upon each of a large number of individuals from a homogeneous population (cf Crozier (1935–36))

The index of dispersion of I_1 or of F_1 is a reproducible property   The two sets of determinations at $F = 25$ illustrate this   We have also made a series of 12 additional sets of determinations of I_m and of $P E_{I_1}$ at $F = 25$, using 6 individuals, the mean values of I_m from these sets was 0 03943 millilambert, agreeing admirably with the values 0 03998 and 0 04061 previously obtained at $F = 25$ (see Table V), $P E_{I_1}$ was found to be 0 002735, the previous values (Table V) being 0 002095 and 0 002939   The various determinations of I_1

at $F = 25$ total in all 95   The frequency distribution of these is quite random (Fig 2), there is no evidence to support the notion that the data are influenced by an "interval of uncertainty" in the flicker effect—as, if this were the case, the mode of the distribution should be well to the left

The points at the lowest intensities used are from the standpoint of the observer the most difficult to obtain   6 weeks after the data for mean I at $F = 2$ and $F = 3$ 33 were secured the determinations were repeated with the same individuals This was done primarily because there was a reasonable suspicion that at the times when the first measurements at these frequencies were made the intensity of the source may have been actually too high, by a small amount (due to battery charging on the same circuit), or too variable   It was also desired to see if the observer's technique had improved or changed in the course of several months' day by day occupation with the taking of such readings, so that P E $_{r_1}$ might conceivably be affected *if* this quantity does reflect precision of observation rather than primarily the variation in the excitability of the *Anax* larvae   The redetermined values of I_m, and of P E $_{r_1}$, at $F = 2$ and 3 33, are entered in Table V, and are given in Figs 3 and 6 (solid circlets with "tags")   The indication is that the intensity of the light source was really a little high (hence, read as too low) in the case of the figures first determined at these particular flicker frequencies, this is strengthened by the findings in two other series of measurements discussed in the succeeding paper   The slight displacement given by the second set of measurements does not in any way affect the treatment of the data   The variation of I is in excellent agreement (Fig 6) with the requirements of the plot drawn before these last determinations were made, and shows the absence of any influence upon the magnitude of P E $_{r_1}$ due to change in the properties of the observer

We are interested chiefly in discussing the rational basis for our method of treating the primary data   It is to be noted that the procedure used, based on the properties of the measurements, results in a picture of the variation of performance which is astonishingly consistent with that already obtained in the case of other organisms (*cf* Crozier (1935–36), Crozier, Wolf, and Zerrahn-Wolf (1936–37))

IV

The determinations of mean critical illumination (I_m) and of mean critical flicker frequency (F_m) are given in Table V, and in Fig 3 It is apparent that the F_m curve is continuously higher than that for I_m at fixed F This is the relationship predicted as a necessary consequence of the fact that the law connecting F and I for threshold

response, as given by the determinations, is not a curve, but a *band* with limits defined by a measure of the probability of the departures

TABLE V

Mean critical illuminations (I_m) at fixed frequencies of flicker (F), with P E I_1 (computed as in text) and mean critical frequencies of flicker (F_m) $\pm$ P E F_1 at these mean intensities, three determinations on each of the same 12 individuals (*Anax junius*) at each point

F	log I_m	P E I_1	F_m	P E F_1
per sec	*mill'lamberts*	*± millilamberts*	*per sec*	*per sec.*
2 0	$\bar{4}$ 4882	0 000,017 52	2 144	0 046,22
	$\bar{4}$ 5884*	0 000 020 94		
3 33	$\bar{4}$ 9005	0 000 036 85	3 589	0 058,37
	$\bar{4}$ 9784*	0 000 050,80		
5 0	$\bar{3}$ 3367	0 000 301 8	5 200	0 108 9
6 72	$\bar{3}$ 5376	0 000,188 9	6 918	0 074 19
8 0	$\bar{3}$ 7308	0 000,435,9	8 168	0 059,47
10 0	$\bar{3}$ 8982	0 000 847 5	10 25	0 118 5
12 5	$\bar{2}$ 0649	0 000 453 4	13 00	0 139 1
16 0	$\bar{2}$ 2830	0 001,443	16 73	0 278 2
20 0	$\bar{2}$ 4881	0 001 678	20 80	0 310 6
25 0	$\bar{2}$ 6019	0 002 095	26 22	0 356 2
	$\bar{2}$ 6086†	0 002 939	26 24	0 363 1
	$\bar{2}$ 5958†	0 002 735		
30 0	$\bar{2}$ 7449	0 002,729	31 06	0 346 7
35 0	$\bar{2}$ 8592	0 002,616	35 99	0 396 7
40 0	$\bar{2}$ 9696	0 004 881	41 04	0 357 8
45 0	$\bar{1}$ 0874	0 004 644	46 09	0 295 1
50 0	$\bar{1}$ 3438	0 010 58	51 19	0 277 5
55 0	$\bar{1}$ 7356	0 028 43	56 05	0 232 3
58 0	0 1252	0 073 08	58 69	0 230 4
60 0	0 5759	0 173 4	60 62	0 178 1
61 0	1 8407	6 825	61 42	0 201 7

$F = 2 5$ stripe cylinder
$F = (3 3-10)$ 10 stripe cylinder
$F = (12 5-16)$, and † 20 stripe cylinder
$F = (20-61)$, 40 stripe cylinder
* Repetition of the experiment the lower values of I_m at $F = 2$, 3 33 probably due to a fluctuation in lamp current (see text)
$F = (2-35)$ inclusive, with gear ratio 1 1, †and (40-61) inclusive, with ratio 2 1

of the individual determinations from the means   The relation between the two curves (Fig 3) is of exactly the same sort as that
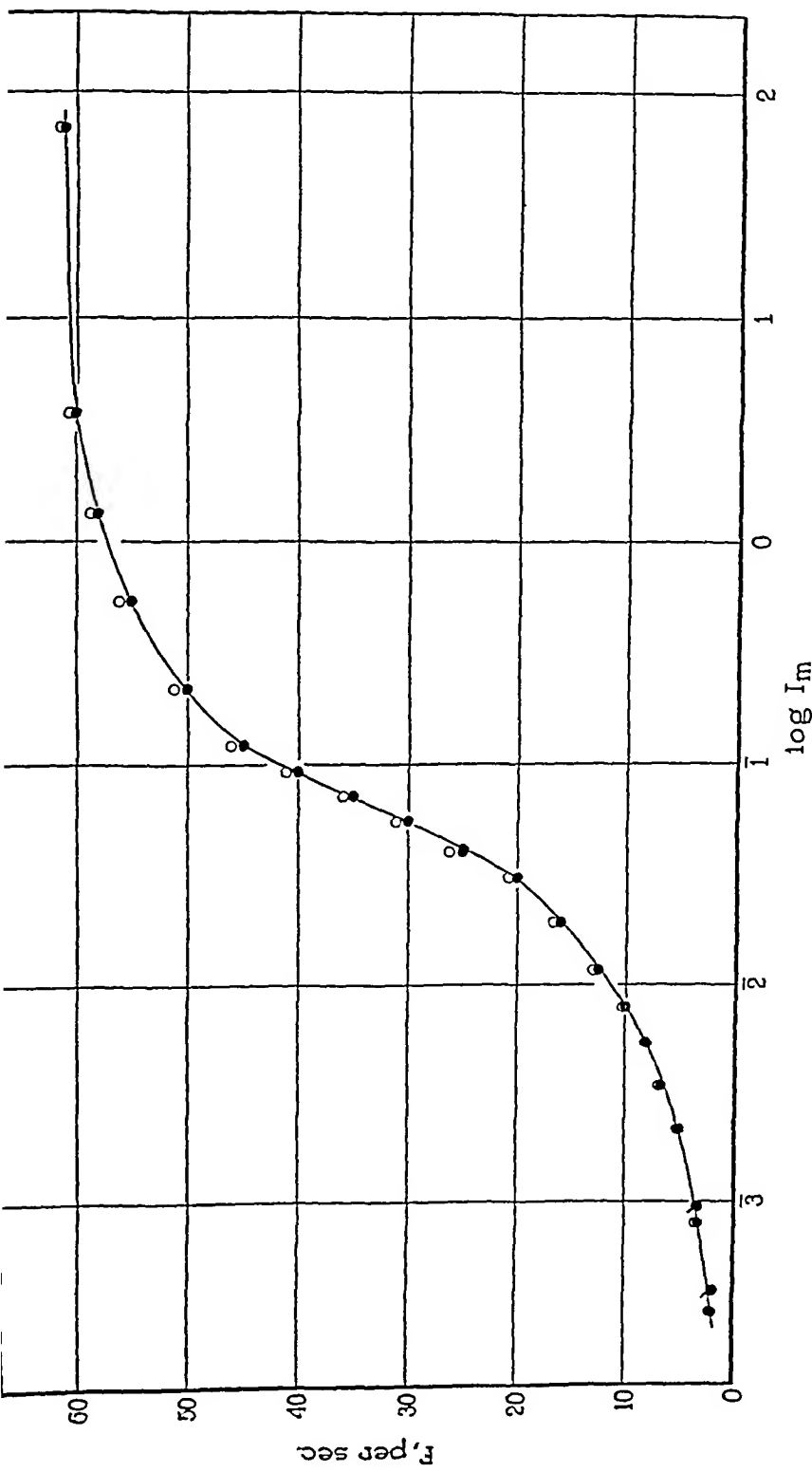
FIG 3 Solid circlets, log I_m as a function of fixed flicker frequencies   Open circlets, F_m as a function of fixed levels of log I_m
See Table V

expected from an analysis of the data on *Apis* (Crozier (1935–36)) and found in the experiments on *Lepomis* (Crozier, Wolf, and Zerrahn Wolf (1936–37))   The present data are superior in the sense that the two curves were obtained concurrently   Calculation from the width of the band describing $I_m \pm P E_I$, as a function of F shows that the discrepancy between them $(F_m - F)$ must be expected to pass through a maximum at the inflection point of the $F - \log I_m$ curve   Fig 4 shows that this is found   The intrinsic lack of precision in the determinations of critical flicker frequency at the highest intensities
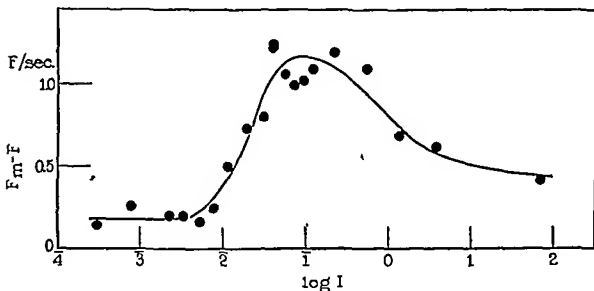
FIG 4 The vertical separation $(F_m - F)$ of the curves based upon (*a*) determinations of mean critical frequency of flicker (F_m) and (*b*) on determinations of mean critical illumination (I_m) at fixed values of F The experimental findings follow a curve which passes through a maximum in the expected way

causes the discrepancy to be larger than expected beyond $F =$ about 80 per cent of maximum F, just as in the data on *Lepomis* (Crozier, Wolf, and Zerrahn Wolf (1936–37))   The difference $(F_m - F)$ is almost exactly $3 \times$ the size of P E $_{r_1}$, and (see Figs 3, 4, 5 and Table V) is *consistent*, the horizontal separation is similarly related to P E $_{f_1}$

We have already referred to the fact that P E $_I$ is lawfully related to I_m The data of earlier experiments with *Apis*, and other relevant material, show that P E $_I$ is directly proportional to I_m Fig 6 exhibits this relationship for the measurements on *Anax*   The several determinations of P E $_{I_1}$ are of the same statistical weight, hence

each is subject to a P E proportional to its own magnitude, consequently a plot of P E $_{I_1}$ vs I_m should be in the form of a band with straight margins which diverge as P E $_{I_1}$ increases (cf Crozier (1929), (1935), etc) For the lower values only of I_m it is mechanically
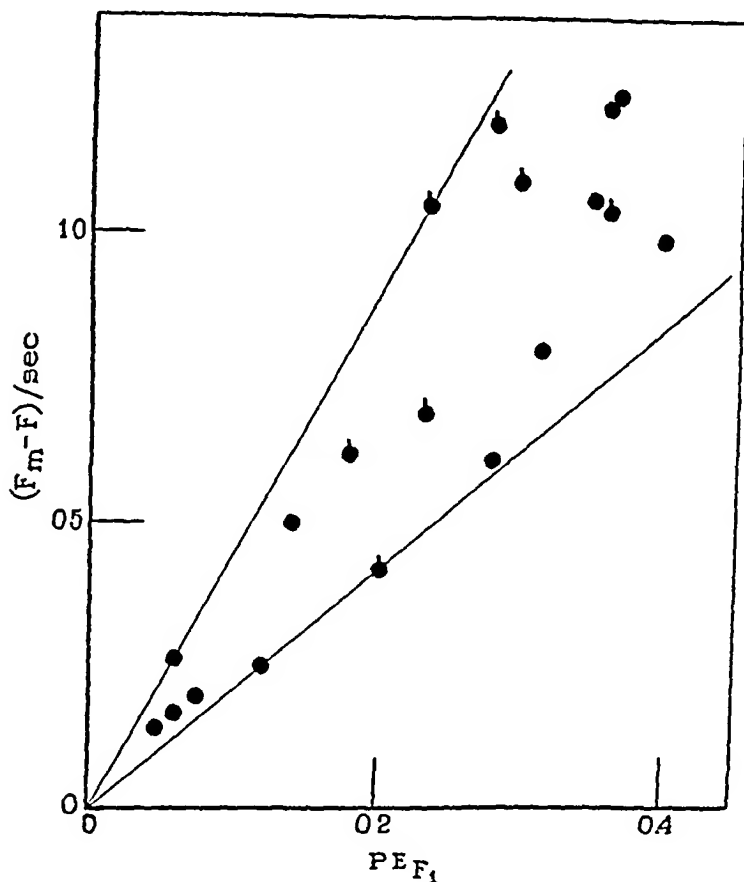

FIG 5 The separation of the curves based upon F fixed and F_m measured (Fig 3) is constantly $= 3$ P E $_{F_1}$ Points above the inflection point in the $F - \log I$ plot (Fig 3) carry tags This separation at any one point could happen once in 20 trials by chance, for the 20 sets of determinations the chance of the separation being accidental is 1 (20)[20]

possible to show this graphically in Fig 7 As is generally the case (Crozier (1935–36)) the band, regarded as straight, does not quite go through the origin, as theoretically it should, the line of central tendency in Fig 5 being

$$\text{P E}_{I_1} = 3 \ 89 \ (I_m + 0 \ 000,008)$$

The "correction," 0 000,008, presumably indicates the magnitude of the constantly present contribution of a source of "error" which is due to the nature of the measurements and is independent of the magnitude
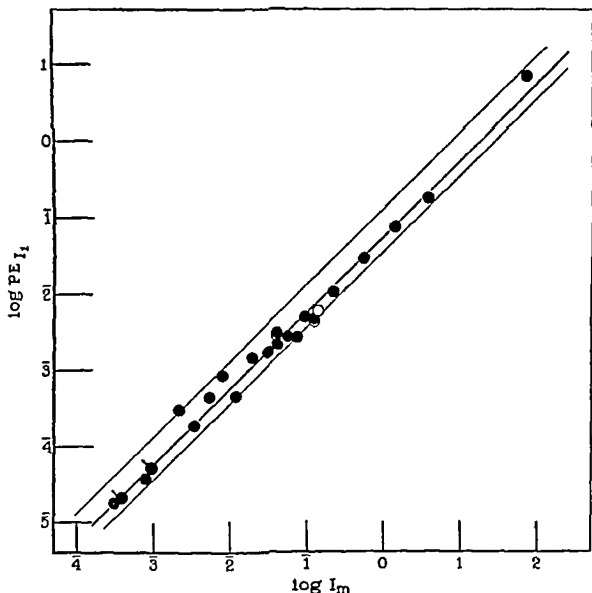

FIG 6 The dispersions of the measurements of I_1, as a function of $\log I_m$, data in Table V 3 additional determinations not included in Table V are plotted as open circlets The points are expected to form a band of constant width The central line divides the band drawn into two arithmetically equivalent zones (bisecting the abscissa dimension) seven observations are above the line eight below it eight on it this demonstrates a random scatter

of I The correction is so small that its effect is not representable in Fig 6 The proportionality of $\sigma P E_I$ to $P E_I$ brings it about that in a logarithmic plotting (Fig 6) the values of $P E_{I_1}$, given a large

number of them, should be distributed in a band with parallel edges
The inflection of the log $I_m - F$ curve (Fig 2) leads to a central
concentration of the plotted points in Fig 6, but the random distribu-
tion of the determinations is attested by the fact that when the band
in Fig 6 is divided into two arithmetically equivalent strips by a
line through the mid-abscissa of its breadth (I_m being the independent
variable) the points are found to be equally distributed on either side
of it (Fig 4)   The reproducibility of the determinations of P E $_{I_1}$
is shown by the "repeat" determination of the point at $F = 25$, al-
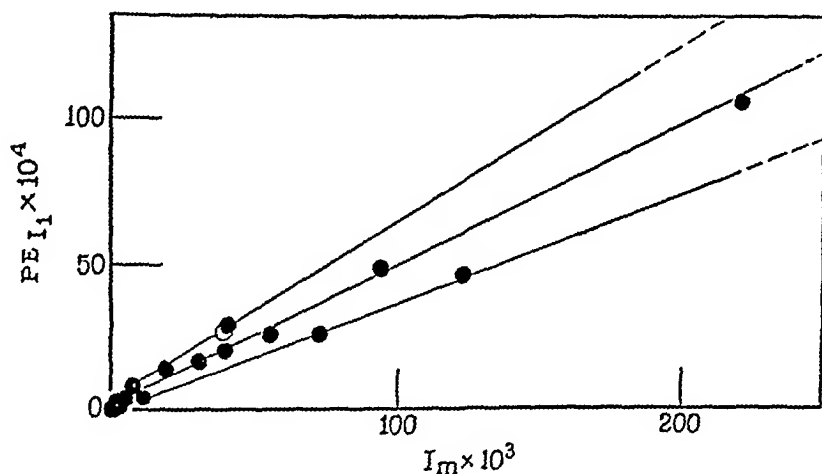ready referred to in Section II, as well as by three supplementary

FIG 7   P E $_{I_1}$ as a rectilinear function of I_m Only the lowest intensities used
can be given on an arithmetic plot   The best fitting line misses the origin by not
more than $I = -0\,000008$ See Fig 6

determinations indicated in Fig 6 by open circlets (not listed in
Table V)

    Just as, from a knowledge of the values of I_m and of P E $_{I_1}$, it is pos-
sible to predict the nature of the curve relating F_m to log I, it is also
possible to predict the form of the dependence of P E $_{F_1}$ upon F_m
(or log I) (Crozier (1935), Crozier, Wolf, and Zerrahn-Wolf (1936–
37))   This is given by one-half the vertical extent of the band formed
by the plotting of $I_m \pm$ P E $_{I_1}$, it rises to a maximum in the region of
the inflection of the log I curve, then falls (this is not *dependent* on
the inflection, since no inflection occurs when F is plotted as a func-
tion of I), the high F end is not so low as the left hand limb, because

of the inherent lack of precision, relatively, in the determinations at
this end—which means that there is less difference among the meas
urements with the several individuals, but lower reliability than there
"should" be in the averages for a single individual   The curve in
Fig 8 shows the dependence of $P E_{r_1}$, as measured, upon $\log I$,
and Fig 9 shows the relation to F_m Two supplementary determina
tions, not entered in Table V, are shown as open circlets   The ver
tical scatter of the points also goes through a maximum, as it should,
for reasons identical with those already mentioned in connection with
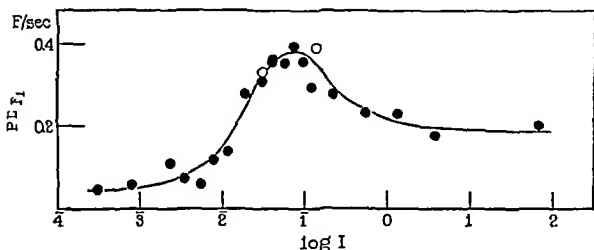the properties of $P E_{r_1}$ (It has sometimes been supposed that in

FIG 8 The dispersion of the measurements of F_i as a function of $\log I$ The
form of the band delimited by $\log (I_m \pm P E_{I_i})$ as a function of F predicts that
$P E_{r_1}$ must pass through a maximum near $\log I \approx 2 85$ The descending limb of
the curve falls to a level higher than that of the left hand end of the curve, for
reasons discussed in the text   Two determinations not included in Table V are
plotted as open circlets

measurements with a biological system the P.E of the measurement
should increase with the magnitude measured, a vague and essentially
irrational analogy being made with the process of repeatedly applying
a foot rule in ascertaining a length   The fact is, of course, that no
such relationship is generally found at all   It is of some value,
however, to point to the behavior of P.E $_{r_1}$ as an instance)
    The correlative behavior of $P E_{i_1}$ and $P E_{r_1}$ shows that the variation
in F_e and in I_e respectively are two aspects of the fundamental organic
variation in capacity for exhibiting the threshold response to flicker
A theory of the mechanism of excitation cannot be based upon the

consideration merely of mean critical illuminations as a function of flicker frequencies, or upon measurements of mean critical flicker frequencies, the fact that these two curves are not the same, and differ in the ways we have discussed, suffices in itself to show that the relation between intensity and flicker frequency, for threshold response, must be stated in terms recognized as preserving the primarily statistical properties of the data. Otherwise dimensional incongruities are inevitable.
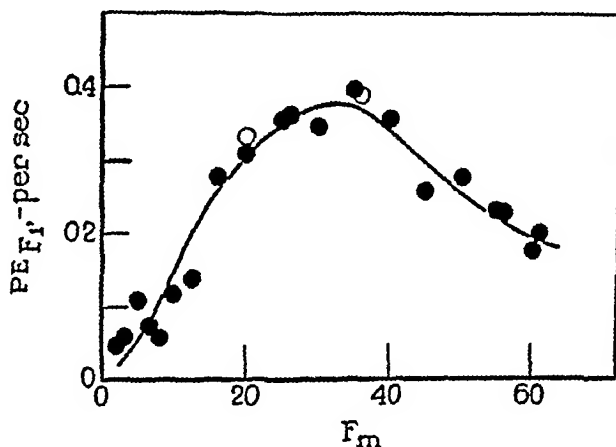

FIG 9 The dispersions of the measurements of F_1 as a function of F_m See Fig 8

V

The reactions of larval Odonata to flickering illumination have been utilized by Salzle (1932) His data of *Aeschna* exhibit in a general way the sort of relationship between F and I_m which we have described for *Anax* The curve is much steeper, however The reaction employed as test of excitation was the "catching motion" of the labium, directed at a moving spot of light on a revolving dark circle The recognition of (that is, reaction to) flicker involves therefore in this case not equal durations of light and dark periods, as in our experiments, but a much greater relative duration of the dark interval It will be shown that this may be expected to make the curve of log I_m as a function of F very much steeper The shape of Salzle's curve, consequently, is not primarily due to a difference in the species used nor in the criterion of excitation Our curve of I_m for *Anax*

is of precisely the same form as that secured in measurements with *Apis* (Wolf (1933–34)) when the light and dark intervals are also equal

Hecht ((1934), Hecht and Verrijp (1933–34), Hecht, Shlaer, and Smith (1935)) has given a provisional theoretical derivation of the equation for flicker fusion with equal dark and light periods It is obtained on the assumption that the change of light adaptation pro ceeding during the light flash and the change of dark adaptation proceeding unopposed during the equally long interruption of light are brought into equality at flicker fusion This cannot be made applicable to flicker fusion specifically without the introduction of an additional assumption, namely that the flicker frequency at fusion is proportional to the amount of photoproduct during a light flash The relation of f, the critical fusion frequency, to intensity should accordingly be given by

$$\frac{2k_1}{k_2} = KI = \frac{f}{(c-f)^m} \tag{1}$$

where k_1 = the photochemical velocity constant, k_2 = the velocity constant of the regenerative dark reaction, c = a constant, the maxi mum flicker frequency, and n and m are exponents signifying the orders of the dark and of the light processes respectively

These equations do not fit the measurements with *Anax*, nor those with *Apis* There should be no significant difference due to the use of F, the frequency for marginal recognition of flicker, instead of f for flicker fusion The fit can be tested by means of Fig 10, we use only the measurements of I_m at fixed flicker frequencies in this illustra tion Log F should be a smoothly increasing function of log I, with continually diminishing slope The slope at the lower end defines exponent n in equation (1), the form of the bend defining m It cannot be said that with $n = 2$, $m = 1$, there is even an approxima tion to the data Increasing m makes the departure worse Even with $n = 2$ the fit at the lower end is not satisfactory It is to be remembered that (Fig 3) there are really two essentially concordant sets of measurements, the departures even at the low end of the graph in Fig 10 are quite significant Moreover, the data on *Apis* have the same properties This cannot be the result of the difference between

"fusion of flicker" as used in obtaining the data described by equation (1) and "marginal recognition of flicker" used in the present cases This is definitely shown by the fact that our data on *Lepomis* (Wolf and Zerrahn-Wolf (1935–36a), Crozier (1935–36), Crozier, Wolf, and Zerrahn-Wolf (1936–37)), based on marginal reaction to flicker, not fusion, are quite well described by equation (1), as Fig 11 demon-
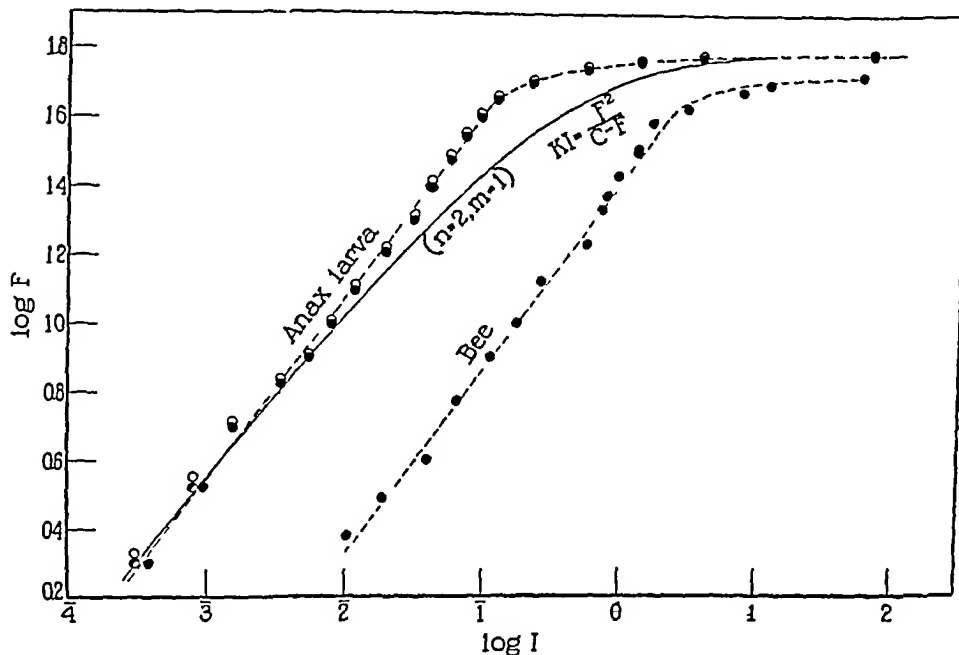

FIG 10 Mean critical illuminations for response to flicker, as a function of fixed flicker frequencies, for *Anax* larvae and for the bee (*cf* Wolf (1933–34)) A logarithmic grid is used to exhibit the way in which the data behave with reference to the applicability of the equation $KI = F^n/(C - F)^m$ The continuous curve is drawn with $n = 2$, $m = 1$ The data for *Anax* and for the bee clearly exhibit the same general properties

strates, in this case, somewhat unusually (*cf* Hecht, Shlaer, and Smith (1935)), $n = 2$ and $m = 1$ for the cone portion

The failure of equation (1) to describe the data from the responses of insects to flickered light might be due to a real mechanical difference in the conditions The curve in Fig 10 might be regarded as in a measure resulting from the inability of low intensities to stimulate a certain proportion of the ommatidia—say those at the periphery of the eye—within the times permitted even at low flicker frequencies

Higher intensities might therefore recruit additional elements The intensity discrimination data on the bee (Wolf (1932–33), *cf* Crozier (1935–36)) contain no hint of such a complication (*cf* Hecht (1935), where $n = m = 2$), it is very doubtful that there is any difference in the technic in the two cases—for example, in the relation of the striped field to the bee's eye—which could account for this, and no obvious trace of any such effect appears in the data on dark adapta tion as followed by means of tests involving intensity discrimination
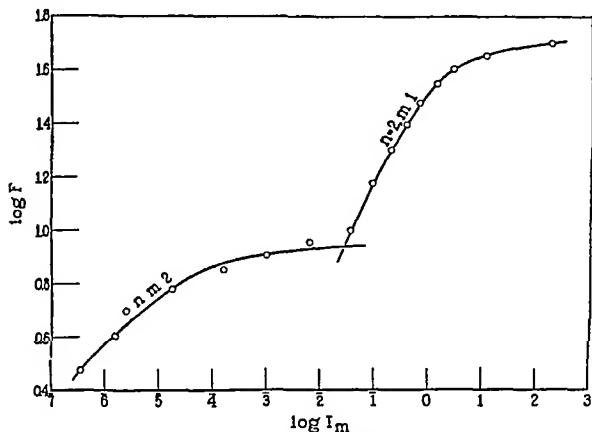

FIG 11 The data on *Lepomis* (Wolf and Zerrahn Wolf (1935–36*b*)) are quite well described by equation (1) see text

(Wolf and Zerrahn Wolf (1935–36*a*), *cf* Crozier (1935–36)) The visual acuity data on the bee, however, with normal eyes and with partially covered eyes (Hecht and Wolf (1928–29)), show an identical break at precisely the intensity at which it occurs in the logistic plot (Fig 13) of the bee flicker data The difference in the shape of the eye in *Apis* and *Anax* also renders this doubtful In any case, the possibility is open to test by means of experiments with only parts of the eye functioning, the visual acuity data do not include measure

ments with only the central area of the eye functioning It is scarcely desirable to assume more than one class of excitable elements   Perhaps the strongest evidence against this is found in the absence of any indications in the data that the variation of I is a discontinuous function of I_m, in the case of *Lepomis* (rods and cones) this evidence is provided (Crozier (1935–36)), but with *Anax*, as with *Apis*, there is no sign of it   We are therefore reluctant to consider that here there are two kinds of receptor elements with organically distinct ranges of thresholds for excitation

Even if by means of a correction for an effect of this sort the measurements with *Anax* and with *Apis* could be made to conform to equation (1) it would not of necessity follow that the assumptions leading to this formula are really efficient   It is implicit in our discussion of these matters that an adequate theory of the situation cannot in fact be based upon measurements of average intensities, because the organism's responses exhibit intrinsic variation (*cf* Crozier (1935–36))   The deduction from other cases involving intensity discrimination is (Crozier (1935–36), and a subsequent paper) that discrimination results from the comparison of the effects produced by two populations of excited elements, or in one population at successive times   Curves of $\log I_m$ as a function of F might therefore be expected to have certain of the properties of population curves   The most generally useful such curve is perhaps the "logistic,"

$$Y = \frac{A}{B + De^{-px}} \tag{2}$$

Equation (1), with $n = m$, is in fact this equation, with

$$F = \frac{F_{max}}{1 + be^{-p \log I_m}}, \tag{3}$$

as then

$$p \ln I = \ln \frac{bF}{(F_{max} - F)},$$

in each case K in (1) $= (1/b)^p$

For cone critical frequencies in the human subject Hecht (Hecht, Shlaer, and Smith (1935)) found $KI = f^2/(c\text{-}f)^2$, so that $p = 0\,5$

When equation (1) describes the flicker curve, as in the case of *Lepo mis*, the logistic does so equally well (Fig 12)   For the cone portion of these data, where we have made $n = 2$, $m = 1$ in (1), the upper portion at least (all that is open to inspection) is well fitted (Fig 12) with p in (3) $= 0\,82$ (cf Crozier (1935–36),[1] for the rod portion, $n = m = 2$ For the asymmetrical case, $n = 2$, $m = 1$, we write
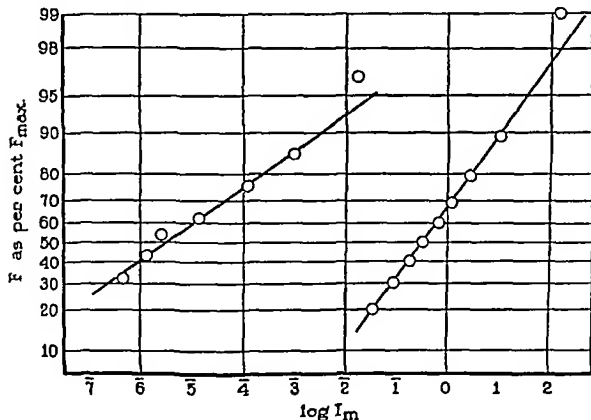
$$F = F_m/(1 + Fe^{-x\,\log I})$$

FIG 12   The data on *Lepomis* are adequately described by the logistic equation, F (as percentage of maximum F) is plotted on a logistic grid (cf Wilson (1921)) as a function of $\log I$ The rod and cone sections are regarded as separate populations, the departure of the highest point could be reduced by adjustment of the assumed value of F_{max} here taken as the highest F observed.   The highest point on the rod curve presumably is a result of overlapping of rod and cone populations

With the data for *Anax*, and for *Apis*, the case is not quite so sim ple (Fig 13)   There is again indication of a change in the relation ship with increasing intensity (incidentally, the transition does not coincide with, and thus seems not to determine, the maximum in the curve of σF_1)   This means that the required form of curve is

[1]p 519

asymmetrical, for example, if in the lower portion of Fig 13, up to $\log I_m = \bar{1} \, 0$, $\sqrt{F}$ is substituted for F the logistic relationship is obeyed very well
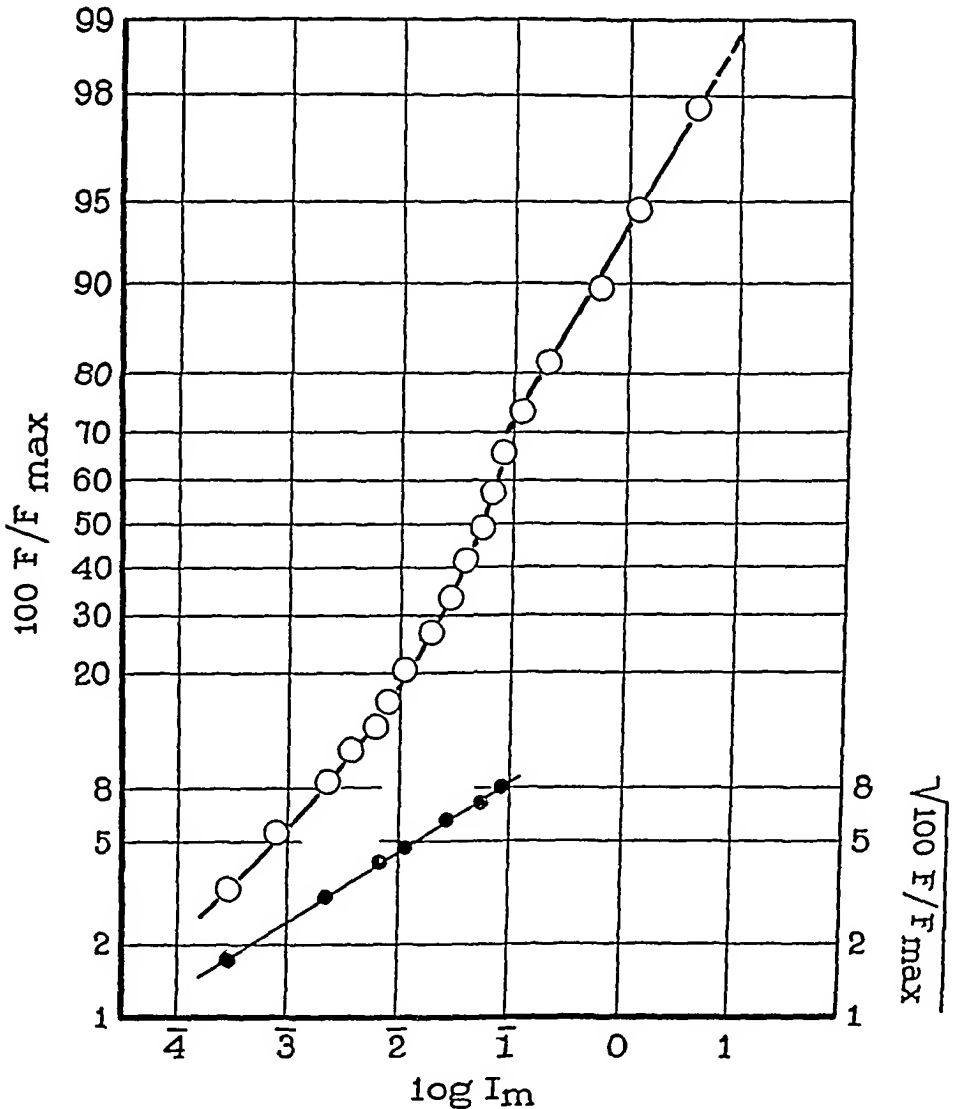

FIG 13 The data on *Anax* show in terms of a logistic grid a separation into two sections, the upper may be regarded as a passable fit ($p = 2$), the lower becomes so if for F/F_{max} there is substituted $\sqrt{F/F_{max}}$ See text

There is thus no reason to suppose that applicability of equation (1) justifies the conception that critical illumination for flicker, as

a function of flicker frequency, *depends upon* the equality of average rates (or amounts) of change of photoproducts in the retina during light adaptation and dark adaptation respectively occurring in the periods of illumination and of no light   It necessarily *involves* such equality, as in any steady state   What is really made questionable by the "population curve" character of equation (1) is the idea that $F (= 1/t)$ corresponds to the amount of photochemical change in a light flash   In fact, however, it can be shown in another way, quite in keeping with the requirements of the basic notion of comparison between "populations of effects" during the periods of light and of no light, that the formal adequacy of equation (1) does not signify the propriety of the reasoning whereby it was arrived at

The constants k_1 and k have the meaning of velocity constants, k_1 for the photochemical change (Hecht (1919–20)) must have a low temperature coefficient (even with allowance for effects upon absorption of light), whereas k_2 must have a much larger temperature coefficient (*cf* Hecht (1926–27), Crozier and Wolf (1928–29))   If we lower the temperature certain specific predictions can be made on the basis of (1)   When the maximum value of F is independent of temperature, and this is the experimentally determined fact in the case we shall discuss, then at fixed flicker frequency F the right hand side of (1) will stay constant, but k_1/k_2 must increase with falling temperature, consequently at fixed F the critical illumination must decrease   Our investigation of this situation is described in a succeeding paper, the general result is that instead of decreasing, the critical intensity *rises* with falling temperature   Consequently k_1 and k_2 (and thus K) cannot have the meanings called for in the derivation of equation (1)   It may also be noted that, from the standpoint of (1), the animal at higher temperatures should be *less* light adapted on exposure to light, consequently, at fixed I, F should fall (*cf* also Lythgoe and Tansley (1929)), actually it rises   The facts thus far considered are, however, in agreement with the requirements of the idea that for flicker fusion there must be established a relationship between F and I such that the average effect of a light flash (or the total effect during the light interval) just fails to be distinguishable from the effect due to or expressed as the after image of the flash   Hecht has pointed out (Hecht, Shlaer, and Smith

(1935)) that the interval between flashes may be occupied with the 'effects governed by removal of photoproducts produced during the flash, not to be confused with the process governing the rate of dark adaptation It is known that in a general way, and over an interval of some seconds, the brilliance of an after image declines, these phenomena are complex, but measurements at least show that when the intensity is gauged by contrast with measured intensities (*cf* Lasareff (1923), Kravkov (1924)) the brilliance declines logarithmically with time

These measurements involve time intervals longer than those concerned in critical flicker frequencies Data do exist, however, making it possible to push back the decay curve to very brief time intervals Fry (1934) measured the photic energy required in the human eye to match the after image of a flash of fixed energy as a function of time after cessation of the flash His figures show that the energy required for a "match," under such conditions that the after image of the first flash persists, declines logarithmically as a function of time (0 0025 to 0 125 second), also that it declines less rapidly when twice the area is initially excited Fry (1934) interpreted his data as indicating inhibition of the effect of a flash by the action of a succeeding flash, this seems to be an unnecessary assumption and indeed without any real foundation These measurements have been utilized by Piéron (1935) to support the theory of "meta contrast" (Stigler (1910), (1918)) Stigler appealed to Exner's conception of the metaphotic image, which Piéron rejects because of the delay in subjective recognition of the onset of a luminous flash, it does not appear to have been realized that these delays in recognition play no part where successive flashes are involved—as indeed the clear demonstration (Rubin (1929), Piéron (1935)) of the erroneous nature of the interpretation of the measurements of *"Empfindungszeit"* (Frolich, 1929) makes evident

There are phenomena of photic response in insects which indicate at least delay in the expression of the effect of a flash (Mast (1912)) It may also be safely assumed that the establishment of the effect produced by a flash does not instantaneously attain its full value, particularly when the flash involves movement of a bar or sector of light For purposes of qualitative illustration and exploration, then, we may assume a scheme of the possible relationships which calls for a decay of the after effect of a flash which is dependent upon (1) the level of effect attained by a flash, a function of its intensity and of its duration, and (2) the control of the rate of decay by temperature, and which regards the establishment of a certain difference between the integrated effect of a flash and of its after effect as necessary for

marginal recognition of flicker   The increase of I at fixed F, when the temperature is lowered, is a necessary outcome of this formulation Certain other consequences of this position, akin to that advocated by Exner (1870) and by Grunbaum (1897–98), will also be dealt with in a succeeding paper

VI

SUMMARY

Curves relating flicker frequency (F) to mean critical illumination (I_m) for threshold response to flickered light, with equal durations of light and no light intervals, and relating illumination (I) to mean critical flicker frequency (F_m) for the same response, have been obtained from homogeneous data based upon the reactions of dragonfly larvae (*Anax junius*)   These curves exhibit the properties already described in the case of the fish *Lepomis*   The curve for F_m lies above the curve of I_m by an amount which, as a function of I, can be predicted from a knowledge either of the variation of I_m or of F_m The law of the observable connection between F and I is properly expressed as a band, not as a simple curve

The variation of I_m (and of F_m) is not due to "experimental error," but is an expression of the variable character of the organism's capacity to exhibit the reaction which is the basis of the measurements As in other series of measurements, P.E $_I$ is a rectilinear function of I_m, P E $_F$ passes through a maximum as F (or I) increases   The form of P E $_F$ as a function of I can be predicted from the measurements of P E $_I$

It is pointed out that the equations which have been proposed for the interpretation of curves of critical flicker frequency as a function of intensity, based upon the balance of light adaptation and dark adaptation, have in fact the character of "population curves," and that their contained constants do not have the properties requisite for the consistent application of the view that the shape of the $F - I$ curve is governed by the steady state condition of adaptation

These curves can, however, be understood as resulting from the achievement of a certain level of difference between the average effect of a light flash and its average after effect during the dark interval

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TEMPERATURE AND CRITICAL ILLUMINATION FOR REACTION TO FLICKERING LIGHT

I Anax Larvae

By W J CROZIER ERNST WOLF, AND GERTRUD ZERRAHN WOLF

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I

Properties of measurements of the relationship between illumination and flicker frequency for response ("recognition of flicker") show that the theory of the relationship must be based upon something more than curves of critical frequency or of critical intensity   Such measurements are not adequately described by their average values [1]   The data really describe the probability that, with a given illumination (I) a certain flicker frequency (F_m) will be found, for a given organism, or conversely, with a given value of F, the probability that a certain critical illumination (I_m) will be found to obtain   The probabilities may be measured by the standard deviations of the observed values of F_1 or of I_1 The indices of dispersion of the observations, with constant numbers of observations, are expressions of a property of the reacting system, and under suitable conditions are not in any sense measures of "experimental error" as ordinarily understood, their primary properties show them not to result from inadequacies or irregularities of technic or of observation   From a knowledge of (1) $I_m \pm \sigma_{I_1}$ as a function of F, the curve (2) of $F_m \pm \sigma_{F_1}$ as a function of I can be predicted in its essential differences from (1)   The law connecting F and I for critical response is thus to be regarded not as a curve, but as a band defining a region within which there is a certain probability that observations of marginal recognition of flicker will occur   This band may not be very broad, so that it might be considered that a curve could be put through it which adequately char

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[1] Crozier (1935-36), Crozier, Wolf and Zerrahn Wolf (1936-37a b)

acterizes the underlying processes responsible for the occurrence of response to flicker   The nature of σ_{I_1} and of σ_{F_1} as functions of I (or of F) makes it impossible, however, to employ the same method of determining an adequate fit of curve to data in the 2 cases (determinations of $F_m = \phi_1(I)$ and of $I_m = \phi_2(F)$, respectively), so a choice becomes arbitrary, and since a number of curves may be made to fit a given set of measurements equally well any final decision must rest upon the dimensional properties of the constants which the selected curve contains   This applies with the same force to equations derived from any relevant theory and to purely empirical curves

Equations have been derived which describe with admirable formal fidelity the relationship between mean values of I_1 as a function of F (cf Hecht, Shlaer, and Smith (1935))   We have found it necessary to inquire into this matter, because it is important for the theory which seeks to provide a generally applicable procedure for the unifying treatment of the data of sensory discrimination (Crozier (1935–36))   The position taken is that discrimination depends upon comparison of effects in two excited populations of nervous elements   These elements may be centrally situated or peripheral, presumably they are central, at least in the meaning that primary receptor cells are not immediately concerned in the comparison upon which the manifestation of response (reaction or recognition) rests   The comparison or competition which results in the signaling reaction is essentially upon a statistical basis and behaves as if dependent upon the standard deviations of the effects produced by these populations of elements   The two sets of effects may originate in two discrete sensory fields, or may correspond to successive states of the same peripheral field and its central nervous representation

With flickered light, at the level of marginal recognition of flicker, a distinction is just made between the effects of light flashes and the effects associated with the alternating intervals in which no light is delivered (cf Fick (1879), Grunbaum (1897–98))   We deal with cases in which the light and the dark interval are equal   In an interval of no light the after effect (positive after-image) of a flash decays   The rapid cycle of flashes and interruptions provides a large number of these intervals   Critical response to flicker involves marginal discrimination between the two "populations" of effects   The action of a

light flash, in terms of the effect produced as concerned in the determination of response, unquestionably fluctuates, it must be supposed that the after effect also fluctuates, in part independently In this way it can be understood that I_m and σ_{I_1}, at various fixed levels of F, are rectilinearly proportional and depend upon phenomena of intensity discrimination (Crozier (1935–36))

The equations describing flicker fusion as a function of intensity, for human visual effects, have approached the matter in another fashion, assuming that the critical frequency f is proportional to the concentration x of photoproduct at the steady state determined by the intensity I, and that at flicker fusion the velocity of formation of x is balanced by the velocity of its "dark" removal Hence

$$2Ik_1/k_2 = x^n/(a-x)^m = f^n/(f_{m a} - f)^m \qquad (1)$$

where

$k_1 =$ velocity constant for photochemical production of x,
$k_2 =$ velocity constant for thermal regeneration of photosensitive material from x
$m =$ order of the photochemical reaction
$n =$ order of the dark process
m and $n = 2$ for human cones,
$m = 2$ or $1, n = 1$ for human rods

The descriptive adequacy of this conception for the human reactions (Hecht, Shlaer, and Smith (1935)) and for those of the fish *Lepomis* (Crozier, Wolf, and Zerrahn Wolf (1936–37 b)), successful also in giving an explanation of the basis of Talbot's law (Hecht and Wolf (1931–32)), does not necessarily justify the assumptions leading to its derivation, as we have already pointed out (Crozier, Wolf, and Zerrahn Wolf (1936–37 b)) When $n = m$, equation (1) is in fact identical in form with the logistic

$$F = \frac{F_{m a}}{1 + c e^{-z \log I}}$$

with $z = 0.5$ for $n = m = 2$, for $n = m = 1, z = 1.0$, for $m = 2$, $n = 1, c = 1/\sqrt{F}$ and the curve of F vs $\log I$ is asymmetrical

While this could be made consistent with the population notion, the significance of equation (1) must be determined by the

properties of its contained constants   The simplest test of their meaning is obtained by varying the temperature   In this case we must expect from (1) that since the temperature coefficient of k_1 must be small while the critical increment for k_2 should be large, the effect of lowering the temperature must be to decrease critical I if f is fixed and if f_{max} is not affected (Crozier, Wolf, and Zerrahn-Wolf (1936–37 b))   The actual result is quite the opposite   Moreover, if in a vertebrate the rod and cone portions of a flicker curve require different values of k_1/k_2 and of m and n, a change of temperature should not affect the two portions in the same way or to the same extent, but experiment shows us that they are affected in just the same degree [2]

This experimental finding is readily reconciled with the intensity discrimination theory of recognition of flicker, and is indeed predicted by it   This interpretation also accounts rather fully for the phenomena of variation, which the customary method of treatment ignores completely or dismisses as an unfortunate concomitant of observation   As has been indicated, however, the variation of I_1 and of F_1 is an essential property of the reacting organism, and the lawful character of this capacity to exhibit variation in the magnitude of critical I_1 or

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[2] In the formulation originally proposed (Hecht and Verrijp (1933–34)), where the case $m = 1$, $n = 2$ was discussed, the stationary state conception led to the expression

$$\frac{KI}{2c} = \frac{f}{\dfrac{a}{c} - \sqrt{f}},$$

where $K = k_1/k_2$, a = total amount of photosensory material (100 per cent), f = critical frequency, and $c = \sqrt{2c'/k_2}$, where $c' = \Delta x$ = the constant quantity of x involved in the fluctuation from dark to light at flicker fusion   This, rewritten, is

$$\frac{k_1 I}{\sqrt{8\, c'\, k_2}} = \frac{f}{\dfrac{\sqrt{ak_2} - \sqrt{f}}{2c}},$$

which gives an increase of I at constant f if the temperature falls, but it necessarily implies a value of f_{max} which is a function of temperature, for which there is no evidence   On the other hand, if c' is also taken as a function of temperature, f_{max} should still rise with rising temperature but one is further left in doubt as to the kind of function to assume for c', no definite prediction would be possible concerning the direction of change of I_m with change of temperature

F_1 must be accounted for, after all, the dispersions of the meas urements are one of their primary attributes—to ignore them is scarcely just, and may be simply willful

We discuss first the relation between temperature and the shape and position of the curve for mean critical illumination as a function of flicker frequency with the larva of the dragonfly *Anax junius*   The measurements have been restricted to determinations of I_m and of σ_{I_1}, we have earlier described the relation between these measure ments and those of F_m and of σ_{F_1} as a function of I for these larvae, at one temperature, 21 5° ± 0 4° (Crozier, Wolf, and Zerrahn Wolf (1936–37 b))   The new determinations were made at 12 4° ± 0 4° and at 27 3° ± 0 2°   Physical limitations precluded for the moment making concurrent determinations of F_m Our main purpose was to investigate the change in position of the $F - I_m$ curve with altered temperature in an animal with which the complication of two sets of receptor elements (rods and cones) would not be encountered   The presumptive effect of the "mechanical disadvantage" of the marginal ommatidia of the eye in the reception of photic excitation (Crozier, Wolf, and Zerrahn Wolf (1936–37 b)) should not in this respect inter fere with simplicity of outcome, although it introduces complications in the use of equation (1) for this animal

The results are also of interest as indicating how an obscure and peculiar relationship to temperature may be obtained in the case of a biological process the measurements of which are intrinsically complex from this standpoint and unsuitable for the determination of tempera ture characteristics (*cf* Crozier (1935))   A subsequent paper contains the outcome of similar experiments with the sunfish *Lepomis* (*cf* Wolf and Zerrahn Wolf (1935–36 b), Crozier (1935–36), Crozier, Wolf, and Zerrahn Wolf (1936–37 a))

II

Procedure and technic have been described in our preceding papers (Wolf and Zerrahn Wolf (1935–36 a b)   Crozier (1935–36)   Crozier, Wolf and Zerrahn Wolf (1936–37a, b))   We have used the larvae of *Anax junius* employed in the earlier work (Crozier Wolf and Zerrahn Wolf (1936–37b))   One of the 12 individuals used died at the beginning of this experiment   Another died in the course of it   The previous determination of the relationship between critical illumination (I) and critical flicker frequency (F) was made at temperature 21 5 ± 0 4   At

fixed flicker frequencies (F) mean values of I_c, labelled I_m, were now found at $t = 12\,4° \pm 0\,4°$ and at $27\,3° \pm 0\,2°$ The average value of I_c was obtained for each larva from three readings of the intensity just required to force it to move with the direction of the system of revolving stripes, the mean of these values, I_1, was then averaged for all individuals and recorded as I_m P E $_{I_1}$ is the P E of the dispersion of the individual values I_1, and is independent of the number of individuals if this number is large enough and if the relative sensitivities of the individuals are distributed at random   The reasons for this procedure have been considered at some length (Crozier, Wolf, and Zerrahn-Wolf (1936–37b))

The relative sensitivities of the larvae used are examined by considering their rank-order numbers in the various sets of measurements   The mean values for the 10 individuals used throughout the experiment are randomly distributed and have the same properties as in the series previously analyzed   Tests were made usually with two flicker frequencies on one day   The correlation between individual rank-order position class in the first of two such tests and the mean value of the rank position in the second test is of the sort already found in comparing sensitivities in successive determinations of relative sensitivity among larvae of one lot in which I_1 and F_1 were found on the same day (Crozier, Wolf, and Zerrahn-Wolf (1936–37b))   Rank-order positions on successive days show no correlation whatever, as found previously   We are justified in regarding the populations of I_1 at the various F's as indicating fluctuations in a property of the reacting system responsible for the larva's reaction

The larvae were kept at a desired temperature, for $27\,3°$, $0\,5°$ above and for $12\,4°$, $0\,5°$ below that at which a determination of I_m was to be made, in individual jars in a well regulated thermostat   With some control of the temperature of the dark room only a slight change of temperature occurred in a jar when it was placed upon the exposure stage of the apparatus for the length of time required for a measurement   This was ascertained by control runs in which the jar contained a thermometer   The series at $12\,4°$ was run first, then that at $27\,3°$ These are at about the most extreme temperatures with which it is possible to obtain reliable results

<div align="center">III</div>

The data are collected in Table I   In Fig 1 the curves of I_m as a function of F at $t° = 12\,4°$ and $27\,3°$ are compared with that gotten with these larvae at $t° = 21\,5°$ It is to be noticed that points on the curve at $21\,5°$ are repeatable after an interval of some weeks

The data show, rather surprisingly, that the maximum flicker frequency, where the $F - I_m$ curve has flattened off, is independent of temperature   They also indicate that the intensity at which threshold response occurs is practically independent of temperature when maximum flicker frequency is attained

The direction of the orderly shift in the $F - I_m$ curve with change of temperature is not that which the use of the stationary state equation calls for (cf Crozier, Wolf, and Zerrahn Wolf (1936–37 b))   The animal should be relatively in a more dark adapted state the higher the temperature, since it must be presumed that the temperature coefficient of the "dark" process, k_2 in equation (1), is greater than that for the photochemical velocity constant k_1 Hence at given intensity I_m F should on this basis be found lower the higher the temperature, but

TABLE I

Mean critical illuminations at various frequencies of flicker (F) at three temperatures   *Anax* larvae   Each mean is derived from the average of three determinations on each of the same 12 or 10 larvae   (At 21 5° a number of values of I_m were determined at other frequencies, these are given in our preceding paper (Crozier Wolf, and Zerrahn Wolf (1936–37 b)) and are plotted in Fig 1)

F	log I_m millilamberts, with log $P.E$ I_1					
	12 4 $\pm$04		21.5 $\pm$04		27 3 $\pm$02	
per sec						
2	$\bar{4}$ 8530	$\bar{5}$ 4433	$\bar{4}$ 5884	$\bar{5}$ 3209	$\bar{4}$ 4153	$\bar{6}$ 9744
5	$\bar{3}$ 5267	$\bar{4}$ 3462	$\bar{3}$ 3367	$\bar{4}$ 4797	$\bar{3}$ 0997	$\bar{5}$ 5904
10	$\bar{2}$ 1173	$\bar{4}$ 6830	$\bar{3}$ 8982	$\bar{4}$ 9282	$\bar{3}$ 7160	$\bar{4}$ 3111
16	$\bar{2}$ 5437	$\bar{3}$ 2573	$\bar{2}$ 2830	$\bar{3}$ 1593	$\bar{2}$ 0795	$\bar{4}$ 8849
25	$\bar{2}$ 8461	$\bar{3}$ 5250	$\bar{2}$ 6019	$\bar{3}$ 4682	$\bar{2}$ 4613	$\bar{3}$ 1790
35	$\bar{1}$ 0795	$\bar{3}$ 9446	$\bar{2}$ 8592	$\bar{3}$ 4166	$\bar{2}$ 6885	$\bar{3}$ 4732
45	$\bar{1}$ 3109	$\bar{3}$ 6035	$\bar{1}$ 0874	$\bar{3}$ 6669		
50	$\bar{1}$ 5417	$\bar{2}$ 4440	$\bar{1}$ 3438	$\bar{2}$ 0245	$\bar{1}$ 1501	$\bar{3}$ 7608
55	$\bar{1}$ 9751	$\bar{2}$ 6973	$\bar{1}$ 7356	$\bar{2}$ 4538		
60	0 6106	$\bar{1}$ 2586	0 5759	$\bar{1}$ 2390	0 5339	$\bar{2}$ 9784
61	1 8532	0 0738	1 8407	0 8341		

the reverse is true   From equation (1), moreover, if F_{max} is independent of I, then at fixed F, I must decrease when k_1/k_2 is increased by lowering the temperature   Since the shift is in the opposite direction the constant k_1/k cannot have the meaning which equation (1) calls for   We have obtained a precisely similar result in experiments with the fish *Lepomis*, discussed in a following article   The finding has therefore an apparently general significance

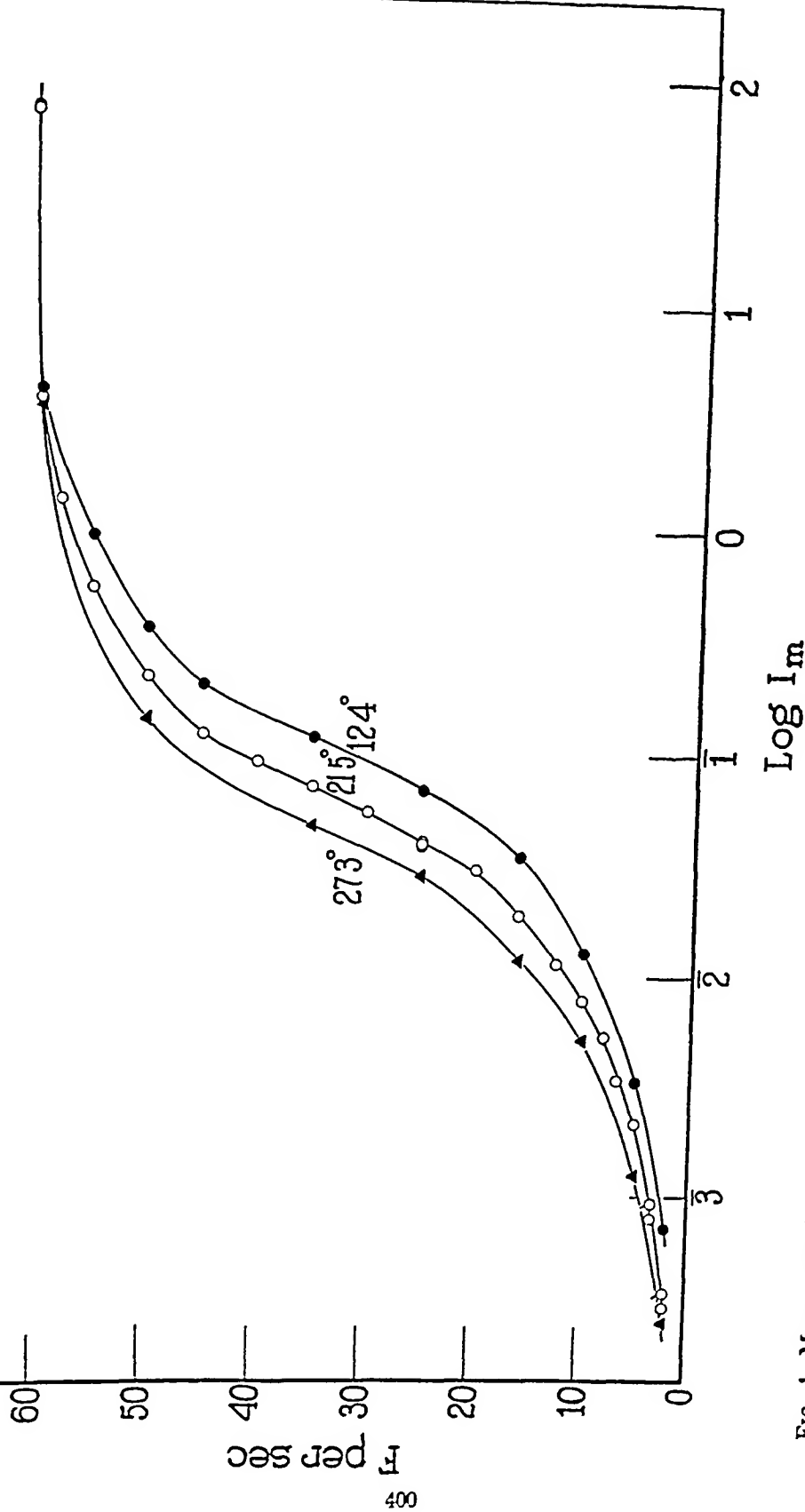The shape of the $F - I_m$ curve is not greatly different at the several

FIG 1 Mean critical intensities for response of *Anax* larvae to flickering illumination, plotted as log I_m, as a function of frequency of flicker (T), at three temperatures (Table I) Data for 21 5° taken from a preceding paper (Crozier, Wolf, and Zerrahn-Wolf (1936–37b))

temperatures   Without undue violence to the data the portion of the curve from $F = 2$ to $F = 20 +$ may be regarded as rectilinear upon a logarithmic grid (Fig 2), the slope is about the same at each temperature $(F = AI_m^{0.324})$ The small deviations from this rule, suggested by the continuous lines drawn through the plotted points, are conceivably consistent and are perhaps real   Above $F = 20$ the curvature is of a different sort and depends upon the temperature, being less
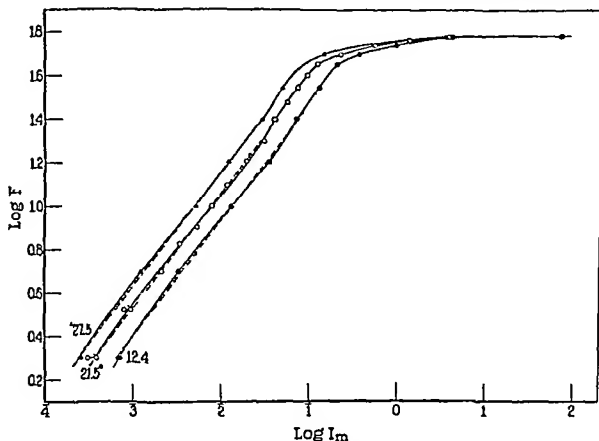
FIG 2   Log I_m as a function of log F The broken lines indicate that up to about $F = 25$ the data may be regarded as signifying that $d \log F/d \log I_m = 0.524$ As the continuous lines suggest however, the deviations may be systematic Above $F = 20$ the curvature depends on the temperature

abrupt the lower the temperature   These relationships also appear when F is plotted against I arithmetically

In the analysis of these effects it might be tempting to regard response at a fixed value of F as corresponding to a constant level of effect achieved by the stimulating agent   It is clear, however, that this is not permissible in any simple sense   The complexity of the matter is shown by the fact that the temperature coefficient of I_m is a

function of F above $F = 20$ and declines as F increases, below this level it is approximately constant, with an apparent temperature characteristic for $1/I_m$ of $\mu = 11,000$, but a value of μ for F at constant I is only 6,000   The temperature characteristic plot, moreover, is not rectilinear in any case, and the apparent temperature characteristic is a function of the temperature

At constant I, F is increased as the temperature rises, the time required for a given intensity to build up an effect such that its decay in an equal period of no light is not detectable, is thus reduced   Similarly, at fixed F, I is reduced as the temperature is increased   But this is not to be taken as evidence that the effect produced by light of given intensity in a fixed time has an appreciably high temperature coefficient   The basis for a *detectable* difference between light flashes and intervals of no light is also a function of the temperature

That up to $F = 25\pm$, $d \log F/d \log I$ is approximately constant (Fig 2), with the value 0 52, independent of temperature, follows simply from the fact that the logistic formulation of the curve (Crozier, Wolf, and Zerrahn-Wolf (1936–37b)) fails in this portion although approximately obeyed if $\left(\dfrac{100\ F}{F_{max}}\right)^{0\,5}$ be substituted for $100\ F/F_{max}$

This disturbance we have suggested as due to the fact that at low intensities, up to $F = 20$, within the time allowed for reaction to flicker according to the activity of the most effective ommatidia, the marginal ommatidia are perhaps at a disadvantage in the reception of light, owing to their angular separation (*cf* Baldus (1926))   Increasing F and using the same criterion for the value of I_c results in using higher intensities, the assumption is that then the effective area of ommatidia involved in the threshold recognition of flicker is increased, because, even with the same duration of a flash, the higher intensity employed can act upon a sufficient number of ommatidia in such a way as to be significant for the determination of the response   For the portion of the flicker curve, then, for which $F = AI_m^{0\,52}$, the value of A may well appear as a function of the temperature, it increases as the temperature falls, because the thresholds for photic excitation for the marginal and other ommatidia at fixed F presumably rise with lowering of temperature—since this must bring about a relative enhancement of the stationary state condition in terms of light adaptation

The magnitude of $\dfrac{dI}{dF} = k\,\dfrac{F}{I}$ (Fig 2) should be inversely proportional to I at constant F and independent of $f°$ if the action of the light depends upon a purely photochemical effect
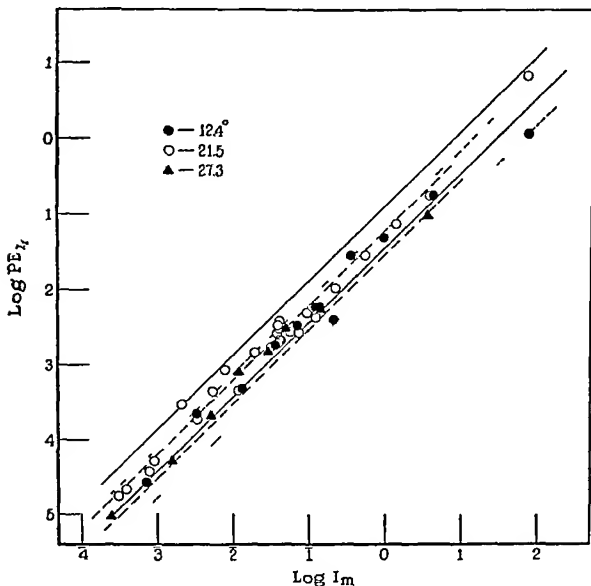

FIG 3 The variation of I_1 measured by P E $_{I_1}$, is a rectilinear function of I_m at each temperature (the slope on the logarithmic grid = 1) The value of the constant B in P E $_{I_1}$ = BI_m changes slightly as the temperature is altered this scatter of the determinations which is proportional to $\sigma_{P\,E\,I_1}$ is discussed in the text

IV

The variation of I_1 obeys the same general rules as in our earlier series P E $_{I_1}$ is a rectilinear function of I_m, log P E $_{I_1}$ is a straight line

function of log I_m, with a slope of 1 (Fig 3) The value of B in the equation $PE_{I_1} = BI_m$ depends to some extent upon the temperature, estimated from the lines of central PE_{I_1} at $\log I_m = 0$, B is 23 4 at 27 3°, 40 5 at 21 5°, 21 0 at 12 4°, and thus appears to go through a maximum at intermediate temperature The spread of the values of $P\bar{E}_{I_1}$, estimated from the vertical widths of the bands in Fig 3, is a measure proportional to $PE_{PE_{I_1}}$, and is thus equivalent to $a \dfrac{PE_{I_1}}{\sqrt{n}}$ where a is a constant and n is the number of I_1's (constant at each temperature) With correction for n, this spread has at $I_m = 1$ the
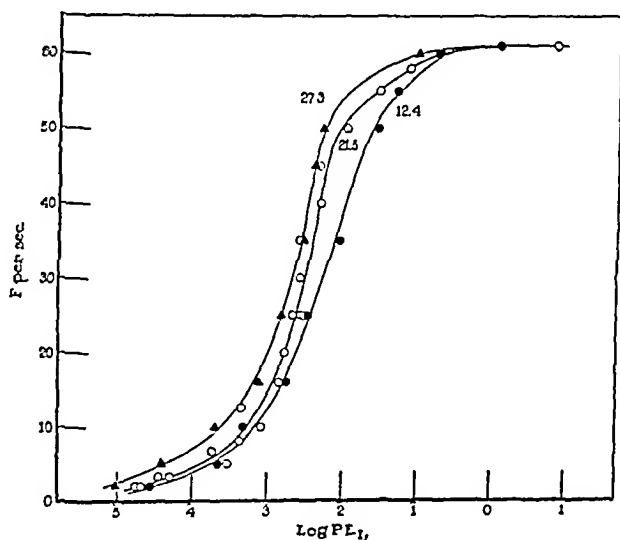
FIG 4 PE_{I_1} as a function of F See text

approximate values 0 107 at 27 3°, 0 222 at 21 5°, 0 204 at 12 4° The proportionality constant for the relation of $PE_{PE_{I_1}}$ to PE_{I_1} has therefore values in the ratios 4 22 8 99 2 52 at these temperatures With all allowance for the difficulty of accurately determining the widths of the bands in Fig 3 it is apparent that the "spread coefficient" is less at the highest and lowest temperatures In consequence of this, PE_{I_1} is also an inverse function of temperature at constant flicker frequency (Fig 4), but the displacement of the curve of PE_{I_1} vs F with change of $t°$ is not quite of the same form In this respect also, then, the law of the connection between F and I_m is equally apparent in the relation of PE'_i to F (Crozier (1935–36))

In addition to demonstrating anew the lawful character of the variation in the determinations of I_1 the additional features suggested by the complex relationships to temperature will require consideration in any theoretical discussion of the basis for discrimination of flicker This is reinforced by the fact that similar relationships appear also in our data on *Lepomis*

We have already commented upon the fact that at higher intensities of illumination the sharper reactions of the animals do not reduce P E $_{I_1}$ (Crozier, Wolf, and Zerrahn Wolf (1936–37 *a*, *b*)) A similar conclusion is apparent from the examination of the data at high and low temperature At any given intensity the movement of the larva which constitutes the threshold response is less sharp and easy to note promptly the lower the temperature, this is in no way correlated with the changes in P E $_{I_1}$ at constant I The relation P E $_{I_1}$ to the 2 variables I and t° therefore gives no support to the idea that P E $_{I_1}$ is an expression of "error of observation," it is consistent with the idea that P E $_{I_1}$ measures the fluctuating capacity of a larva to exhibit the discrimination of flicker, and that this fluctuating capacity is an elementary property of the mechanism of the discrimination

<div align="center">v</div>

The change in the curve of I_m as a function of F when the temperature is altered can be accounted for qualitatively on the basis that distinction of flicker requires a discrimination between (1) the effects of flashes and consequent (2) the after effects consequent upon the action of these flashes It cannot be accounted for quantitatively from the form of the F–I_m curve alone, although progress in this direction can be made by taking into account the variation of I_1, additional information is required as to the effect of varying the proportion of "light time" to "dark time" in a flicker cycle, to be obtained in subsequent experiments

We assume that the building up of the effect of a single flash of light does not occur instantaneously, but with a speed which is significant and is a function of I and of the level of light adaptation (*cf* Bills (1920)), that the magnitude of the effect produced at the termination of the flash is essentially a function of the intensity and of the duration of the light period, and that the I process is primarily photochemical and has a small temperature coefficient The after effect of a flash

is assumed to be the result of a "dark" process with typically large temperature coefficient for its velocity of decay (Fig 5)   It is not required at the moment to correlate these assumptions (Crozier, Wolf, and Zerrahn-Wolf (1936–37 *b*)) with the suggestive and presumably relevant observations on the building up and decay of retinal potentials, optic nerve discharges, and cortical potentials (*cf* Adrian and Matthews (1928), Hartline (1925), Granit and Davis (1931), Granit
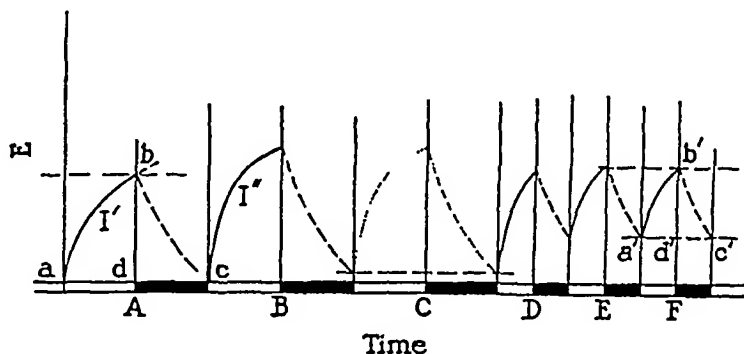

Time

Fig 5   When flicker is just recognized (*A*) the action of a flash (*a–d*) of intensity I' is represented by the curve *a–b*, (*a–d*) $= 1/2\ F$ seconds, *E* is a scale of effect produced   In the succeeding dark interval, of equal length, the effect at *b* decays   Recognition of flicker is assumed due to a certain proportionate difference between areas *a b d* and *b d c*   The curves *a b* and *b c* are assumed first order for convenience   If *F* is kept constant and I' is increased to I'' (*B*), a condition is attainable in which *E* fluctuates between the same levels (dotted lines, *C*), but only at the expense of a larger discrepancy between the "light" and "dark" areas— the flicker is more pronounced   This can be brought back to a condition of marginal response to flicker only by reducing 1/2 *F*—that is, by increasing *F* (as at *D*), when a new condition of balance is attained (at *F*) the same kind of difference between *a'b'd'* and *b'd'c'* can be established as in the base of *a b d* and *b d c* at *A* This corresponds diagrammatically to the fact that as *F* is increased, *I* increases also

(1935), Bartley (1934), etc)   The apparently fixed character of the maximum flicker frequency, and its comparative independence of intensity at different temperatures, presents a special problem   It is a curious fact that in various forms which have been investigated (man, *Lepomis*, bee, *Anax*) F_{max} has values which range only from about 50 to 61 per second   This suggests an interval of about 0 009 second as a general minimum time for photochemical action of a repeated flash permitting recognition of flicker when the light and dark

intervals are equal, above an intensity characteristic of the type of eye briefer flashes are not then reacted to as flickering   The value of F_{max} is, however, dependent upon the area of the flicker field on the retina (Hecht and Smith (1935)) and upon wave length (Hecht and Shlaer (1935)), so that interpretation in terms of anything resembling "implicit time" would appear to be quite dubious   Two additional assumptions are required for the formulation of a mechanism to account for the observed effects   These are (1) that the "after image
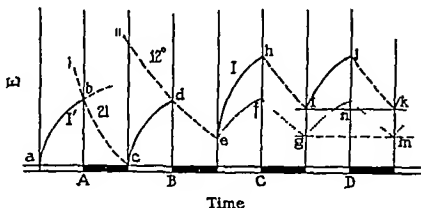
FIG 6 At A a relation between I' and F is depicted at a higher temperature such that (cf Fig 5) flicker is just reacted to on the basis of the difference between the light" area and the 'dark (as a function of the "light" area)   The tempera ture is then lowered (at B), the velocity constant of the dark process governing the decay of E is then lowered, giving a curve of form u in contrast to i keeping I' the same a new condition of constant rise and fall of E (dotted lines) can be attained (at D) but the areal difference is then insufficient for recognition of (reaction to) flicker If, however, the intensity is increased to a value I'' (at C) a condition of balance is obtainable in which the difference between $i j n$ and $j n k$ is great enough, in terms of $i j k$ to permit recognition of the difference   (If I' were retained the same result is to be obtained by lowering the flicker frequency)   These relationships are consistent with the experimental findings

process" does not proceed during a light flash, but that its decay begins when delivery of light is interrupted, and (2) that for marginal recog nition of flicker only the increase and decrease of effect are significant These ideas are embodied in the diagrams given in Figs 5 and 6   Fig 5 shows how for marginal recognition of flicker on this basis, a balance between the effect of a flash (the area $a \, b \, d$) and the effect during the dark interval (area $d \, b \, c$) requires a certain difference between the two areas   We may consider that the difference should be a certain fraction of $a \, b \, d$ If now the intensity be increased from I' to I'', the

areal difference at balance (C) will be too large, unless (at F) the flicker frequency is raised (time of a flash shortened)   The curves are assumed to be first order, for simplicity, the rate constant for the I curve must be assumed less (in these cases only very slightly less) the higher the level of E, to account for the effect of the state of adaptation

In Fig 6 the effect of change of temperature is illustrated   There is necessarily demanded by this formulation an increase of intensity at constant flicker frequency if the temperature is lowered, if the average integrated effect of a flash is just to be distinguished from the average integrated after effect

With higher intensity (and F), at fixed temperature, a given small change of intensity will produce a smaller absolute and relative change in the area $a\ b\ d$ (Fig 5) and in the difference $(a\ b\ d) - (b\ d\ c)$, consequently a larger variation in I will be tolerated and will appear in the measurements

When the temperature is lowered, at fixed F, I_m is higher   But the natural fluctuation in the decay constant of the after effect process will be expected to be less the lower the temperature—as measurements of velocities of biological processes as a function of temperature uniformly show (Crozier (1935))   Hence with fall of temperature we expect at constant I_m (and thus with falling F) that the tolerated variation of I_1 for recognition of flicker will pass through a maximum, as we have seen is indicated, with decreasing F (= increasing time of a flash), the area $a\ b\ d$ will increase, and a given change of I will produce a larger absolute effect, but as the temperature falls this will stand less and less chance of being cancelled by intrinsic variation in the velocity of the after effect decay

It is not difficult, on this basis, to account for the behavior of P E $_{r_1}$ as a function of I (Crozier, Wolf, and Zerrahn-Wolf (1936–37 a, b)), but this need not be gone into in detail here, since we do not yet have direct information as to the changes of P E $_{r_1}$, as a function of I, as the temperature is altered   But we may point out that at fixed $t°$ as I is increased, and F, P E $_{r_1}$ passes through a maximum (Crozier, Wolf, and Zerrahn-Wolf (1936–37 a, b)), consequently a fixed deviation in F, δF, must be relatively constant in its effect in a mid-range of I and of F and must be of relatively greater consequence at the two ends, at high I and F (brief time) it should be easier to detect since it would

(in terms of its influence upon the area $a\ b\ d$ in Fig 5) be more sig
nificant and less likely to have its effect cancelled by the action of
spontaneous variation in the velocity constant of the light excitatory
process, and at low I and F we should expect to find δF more influential
owing to its there producing an effect comparable to slight change in I
With information to be obtained from experiments in which the pro
portion of light time to dark time in a cycle is varied, it should be
possible to make a closer approximation to the forms of the curves in
Figs 5 and 6, and thus to account precisely for the variability relation
ships discovered

<div align="center">V</div>

<div align="center">SUMMARY</div>

The curve of mean critical illumination (I_m) for response to flicker
as a function of flicker frequency (F) for the larvae of the dragonfly
Anax junius is progressively shifted toward higher intensities the lower
the temperature   The maximum flicker frequency (one half the cycle
time of light and no light) and the maximum intensity with which it is
associated are very little if at all affected by change of temperature

These facts are in agreement with the requirements of the concep
tion that recognition of critical illumination for reaction to flicker
involves and depends upon a kind of intensity discrimination, namely
between the effects of flashes and the after effects of these flashes during
the intervals of no light   The shift of the F–I_m curve with change of
temperature is quite inconsistent with the stationary state conception
of the determination of the shape of the curve

The dispersion (P E $_{I_1}$) of the measurements of I_1 is directly propor
tional to I_m, but the factor of proportionality is less at high and at low
temperature than at an intermediate temperature, the scatter of the
values of P E $_{I_1}$ is also a function of the temperature   These facts can
also be shown to be concordant with the intensity discrimination basis
for marginal recognition of flicker

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TEMPERATURE AND CRITICAL ILLUMINATION FOR REACTION TO FLICKERING LIGHT

II SUNFISH

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I

The relationship between frequency of interruption (F) and intensity of illumination (I) for threshold response indicating discrimination of non-continuous light (flicker) exhibits in the case of the sunfish, as in the human (Hecht and Verrijp (1932–33 a, b), Hecht (1934), Hecht, Shlaer, and Smith (1935)), two distinct sections a rather flat low intensity portion assigned to the exclusive or predominant activity of rods, and a steeper portion, rising abruptly from the former, reflecting the involvement of cone vision (Wolf and Zerrahn Wolf (1935–36), Crozier, Wolf, and Zerrahn Wolf (1936–37 a)) The curve of I_m as a function of F differs from that of F_m as a function of I in a manner predictable on the basis (Crozier (1935–36)) that the law of the relationship is properly to be described not by a curve, but as a band based upon the observable dispersions of the measurements (Crozier (1935–36), Crozier, Wolf, and Zerrahn Wolf (1936–37 a)) The curve of I_m as a function of F is quite well fitted, however, by the logistic

$$F = F_m \ /(1 + e^{-\rho \log I}), \tag{1}$$

where the value of ρ differs for the rod and cone sections, or by a log probability integral, or at 21 5° by another form of this equation, with theoretical implications, which has been used to describe the flicker extinction curve in the case of man (Hecht, Shlaer, and Smith (1935))

$$K I = \frac{F^n}{(F_m \ - F)^n} \tag{2}$$

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The equation does not really fit in a satisfactory way at the extreme lower (rod) end   The question arises as to whether this stationary state equation ((2), with $F \equiv \lambda$, the stationary state concentration of photoproduct derived from decomposition of photosensitive material) correctly formulates the theory of the mechanism whereby flicker extinction is attained   There should be no essential difference in the properties of data based upon (*a*) marginal recognition of flicker and (*b*) upon marginal extinction of flicker

The K in equation (2), on the basis that (2) is the stationary state equation, is the ratio of two velocity constants (Hecht, Shlaer, and Smith (1935)),

$$K = 2k_1/k_2,$$

$k_1 = $ the photochemical velocity constant concerned with the production of λ, $k_2 = $ the velocity constant of the thermal "dark" process whereby λ is removed

Equation (2) thus requires that with increase of temperature k_2 will increase much faster than k_1, and consequently that if F_{max} is not affected by temperature then at any fixed F the mean critical illumination must increase

We have tested this in the case of the larvae of the dragon fly *Anax junius*   Instead of increasing with rise of temperature, I_m decreases This result is quite consistent, however, with the requirements of the idea that a certain type of difference between the effect of a light flash and the after effect of its action is required for recognition of flicker (Crozier (1935–36), Crozier, Wolf, and Zerrahn-Wolf (1936–37 *b*, *c*)), and was indeed predicted by the use of this conception, it also gives a reasonable account of the properties of the variation of I_1 It has been desirable to extend these observations to deal with the visual reactions of a vertebrate   If the same type of dependence of I_m and of σ_{I_1} upon temperature should be apparent, as is the case, the possibility of a general theoretical treatment is thereby greatly improved   And the real meaning of equation (1) and particularly of equation (2), will be clearer if in the case of the vertebrate the rod and cone portions of the flicker curve (with their necessarily different magnitudes of K) are found to be influenced by change of the temperature in just the same manner or in different ways

II

The experiments were made with the sunfish previously called *Lepomis Ennea-canthus glorioss* (Holbrook), using exactly the procedure in our earlier work. We are indebted to Mr W C Schroeder of the Museum of Comparative Zoology for the identification of these 'little sunfish" and their separation from the very closely similar *Lepomis* We had previously determined I_m as a function of F at 21 5 ± 0 5 (Wolf and Zerrahn Wolf (1935–36)) This curve was re-determined

TABLE I

Mean rank-order positions (R_I), indicating mean comparative values of I_1 in a series of tests at various values of F, for different individual sunfish $R_F =$ a series based on determinations of F_m the same individuals were used in all tests at 12 4° and 27 3°, another lot at 21 5° Increasing R_I in order of increasing I_1

Sunfish No.	Temperature			
	12 4	21.5		27.3
	$N = 10$ fish	$N = 12$ fish		$N = 10$ fish
	R_I $n = 26$ sets	R_F $n = 23$ sets	R_I $n = 15$ sets	R_I $n = 18$ sets
1	6 13	4 55	6 57	5 94
2	5 87	4 69	4 87	6 88
3	6 13	5 14	7 40	6 53
4	6 75	6 86	7 10	6 14
5	5 27	6 76	7 87	5 03
6	4 02	6 76	7 30	5 28
7	5 50	8 42	4 20	4 97
8	4 61	6 76	5 33	3 92
9	5 17	8 01	7 47	5 50
10	5 15	5 76	7 07	4 47
11		6 91	6 03	
12		7 12	7 23	
Mean ± P.E.	5 46 ± 0 549	6 48 ± 0 846	6 24 ± 0 915	5 47 ± 0 652

at 12 4 ± 0 4° and at 27 3° ± 0.3 These temperatures were chosen for convenient comparison with the results given by *Anax* larvae at the same temperatures (Crozier Wolf, and Zerrahn Wolf (1936–37c)) Several points on the curve at 21 5 were re-determined, and found to agree very precisely with the figures in the earlier series

Ten individuals were employed, the same fish being used throughout the tests With each fish, at each flicker frequency (F), three readings of critical intensity (I) were made, the average of these 3 = I_1 for this individual From the 10 values of I_1 at each F the mean (I_m) was computed and the P.E of I_1

The procedure was that which we have already described (Wolf and Zerrahn-Wolf (1935–36), Crozier, Wolf, and Zerrahn-Wolf (1936–37b, c)) We desired to see how the variation of I_1 would behave at the several temperatures The variation of F_1 is less easy to study in this respect, since the law of σ_F as a function of fixed values of I is not so simple as that of σ_I as a function of I_m (cf Crozier (1935–36), Crozier, Wolf, and Zerrahn-Wolf (1936–37a, b)) Our use of P L $_{I_t}$ is based upon the fact that, as I_1 has been determined, it reflects in our population of individuals a fluctuating capacity to exhibit the index response The relative sensitivities of the 10 individuals are randomly distributed in the successive tests at different values of F, this is taken to indicate that each fish gives, at any time, an *independently random level* of I_1, the several individuals exhibit, at one time, levels of sensitivity such as any one fish would show if examined repeatedly, in combination with the lawful behavior of P E $_{I_t}$ as a function of I_m we are thus

TABLE II

Showing the absence of correlation between relative sensitivities of individual sunfish in successive sets of determinations of I_1 The individual rank-order numbers (R_I) are assigned in order of increasing values obtained for I_c The mean values of $R_{I''}$ in the second of two determinations (same afternoon) associated with $R_{I'}$ classes in the first determinations were

$R_{I'}$	1 5	3 5	5 5	7 5	9 5
12 4° (24 sets, 10 fish)	5 72	6 25	5 30	5 38	4 75
$R_{I''}$ 21 5° (23 sets, 12 fish)	6 47	7 01	5 80	6 93	6 80
27 3° (18 sets, 10 fish)	4 94	5 41	5 21	5 61	5 56

enabled to regard the indices of dispersion as measuring a primary property of the capacity to give the response to flicker This has been indicated already for our measurements at 21 5° It is substantiated by the data at the other two temperatures, as Table I demonstrates It is apparent in Table I that the average position of an individual, as regards relative sensitivity, does not differ by more than is to be expected by chance from the mean of its set, or from that of any other individual, the extreme difference between entries in any column of Table I = 2 3 P E $_{D\nu}$ In successive sets of trials on the same day (Table II) there is no correlation between the two rank-order numbers for the same individual

III

The data are given in Table III, the measurements of I_n at 21 5° (Wolf and Zerrahn-Wolf (1935–36), Crozier (1935–36), Crozier, Wolf, and Zerrahn-Wolf (1936–37 a)) are included for comparison They

are exhibited graphically in Fig 1   The range of intensities covered
is so great that in a plot upon this scale it is difficult to do justice to the
figures

TABLE III

Mean critical intensities (log I_m, and P E I_1) for reaction of sunfish to flicker
ing light as a function of flicker frequency F, at three temperatures   (Data
at 21 5 from Wolf and Zerrahn Wolf (1935–36) Crozier, Wolf and Zerrahn
Wolf (1936–37a)   See text

F	log I_m ± P E I_1 mill lamberts		
	12 4	21 5	27.3
per sec			
3	$\bar{7}$ 9201 ± 0 536 × 10⁻⁷	$\bar{7}$ 6555 ± 0 694 × 10⁻⁷	
4	$\bar{6}$ 3392 0 106 × 10⁻⁶	$\bar{6}$ 1784 0 520 × 10⁻⁶	$\bar{7}$ 9708 ± 0 372 × 10⁻⁷
5	$\bar{6}$ 5594 0 154 × 10⁻⁶	$\bar{6}$ 3701 0 399 × 10⁻⁶	$\bar{6}$ 1775 0 378 × 10 ⁷
6	$\bar{5}$ 3593 0 211 × 10⁻⁵		
	$\bar{5}$ 3784 0 931 × 10⁻⁴	$\bar{5}$ 2385 0 303 × 10⁻⁵	$\bar{5}$ 0253 0 281 × 10⁻⁶
7	$\bar{4}$ 3738 0 165 × 10⁻⁴	$\bar{4}$ 1855 0 279 × 10⁻⁴	$\bar{5}$ 9937 0 217 × 10⁻⁵
8	$\bar{3}$ 1844 0 357 × 10⁻³	4 9954 0 190 × 10⁻²	4 7711 0 133 × 10⁻⁴
9	$\bar{2}$ 0060 0 152 × 10⁻²		
	$\bar{2}$ 0047 0 361 × 10⁻²		
	$\bar{2}$ 0132 0 624 × 10⁻²	$\bar{3}$ 7983 0 124 × 10⁻²	$\bar{3}$ 6732 0 274 × 10⁻²
10	$\bar{2}$ 7638 0 175 × 10⁻²	$\bar{2}$ 5600 0 743 × 10⁻²	$\bar{2}$ 3665 0 846 × 10⁻²
12	$\bar{2}$ 9560 0 339 × 10⁻²		2 5419 0 124 × 10⁻²
15	$\bar{1}$ 1735 0 239 × 10⁻²		
	$\bar{1}$ 2375 0 146 × 10⁻¹	$\bar{2}$ 9543 0 126 × 10⁻¹	$\bar{2}$ 7440 0 236 × 10⁻²
20	$\bar{1}$ 4637 0 160 × 10⁻¹	$\bar{1}$ 2591 0 177 × 10⁻¹	$\bar{1}$ 0592 0 319 × 10⁻¹
25	$\bar{1}$ 7755 0 268 × 10⁻¹	$\bar{1}$ 5631 0 630 × 10⁻¹	1 3609 0 166 × 10⁻¹
			$\bar{1}$ 3602 0 715 × 10⁻¹
30	0 0453 0 278 × 10⁻¹	$\bar{1}$ 8118 0 731 × 10⁻¹	$\bar{1}$ 6249 0 162 × 10⁻¹
35	0 3465 0 120	0 1418 0 129	$\bar{1}$ 9444 0 291 × 10⁻¹
40	0 7308 0 168		
	0 7383 0 151	0 4601 0 281	0 2423 0 620 × 10⁻¹
45	1 2748 0 571		
	1 2728 0 465	1 0465 0 415	0 8532 0 166
48			1 3056 0 558
50	2 2751 1 844		
	2 2817 1 933	2 2264 11 53	2 1898 2 994

We have indicated that the measurements at 21 5° are described
rather well by the equation (Hecht, Shlaer, and Smith (1935))
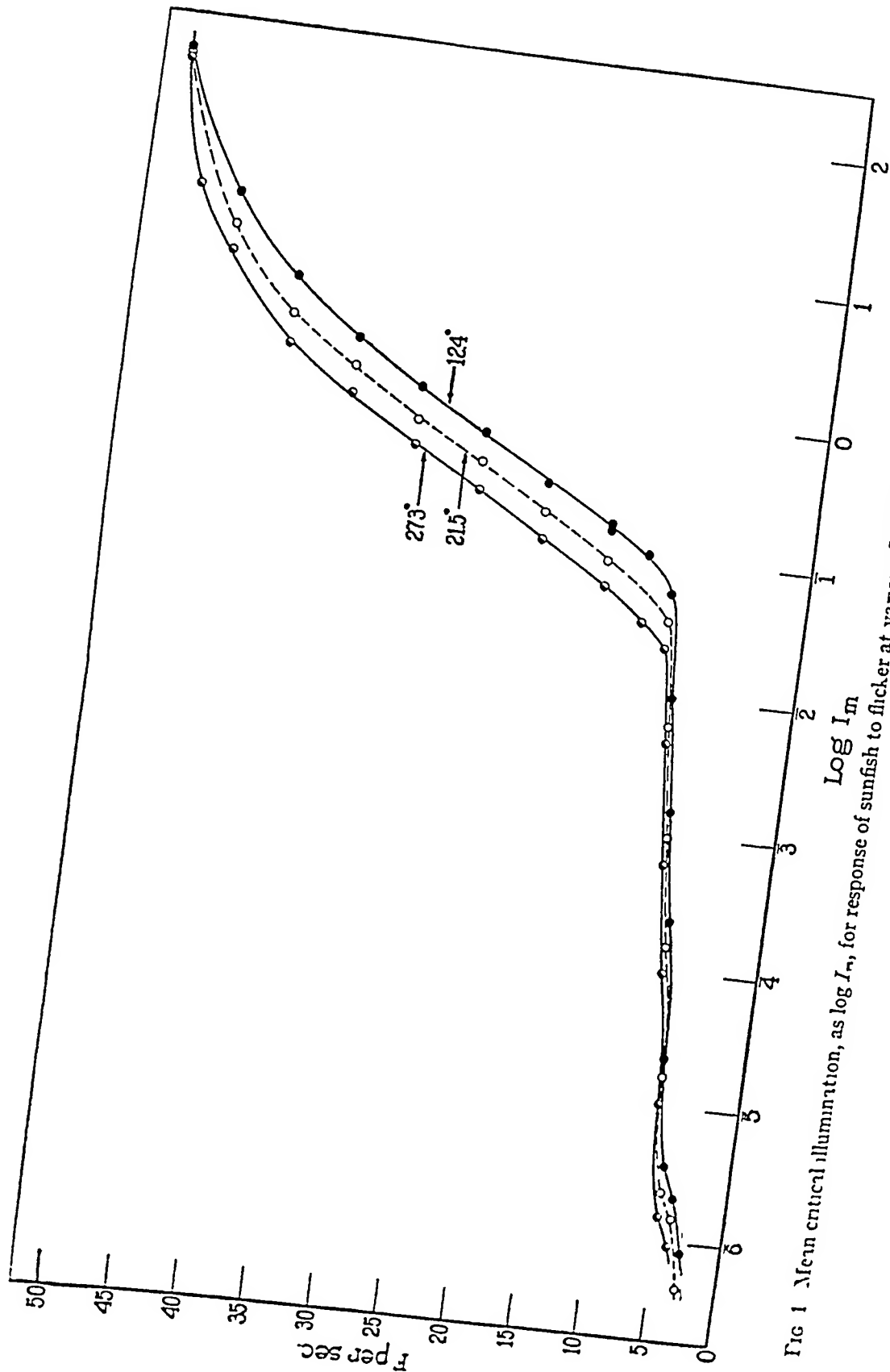
$$KI_m = F^n/(F_{max} - F)^n \tag{2}$$

FIG 1 Mean critical illumination, as log I_m, for response of sunfish to flicker at various flicker frequencies F, at three temperatures

where $n = 2$, $m = 2$ for the lower (rod) segment and $n = 2$, $m = 1$ for the upper (cone) portion (Crozier, Wolf, and Zerrahn Wolf (1936–37 b))   The extent to which this is true of the data at the other two temperatures (Fig 2) may be most conveniently shown in a plot of log F vs log I_m The fit may be considered acceptable for the "cone portion," although with these values of the exponents it is not really adequate at 12 4° or at 27 3°, the curvature is a function of temperature   The deviation at the lowest frequencies is real and significant
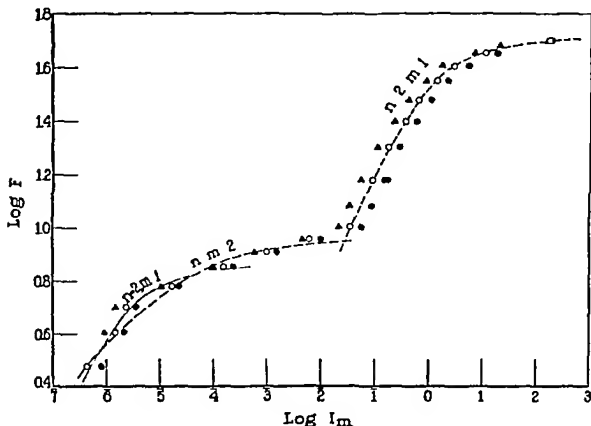
FIG 2   Log I_m as a function of log F See text

(The arrangement of the data at the lower end has earlier been treated as due to chance variation (Crozier, 1935–36))   On the basis of a possible separation of the rod and cone contributions which is discussed subsequently (page 424), the region from $F = 7$ to $\Gamma = 10$ represents a zone of intensities in which the two contributions are added, one could then fit equation (2) to the points at $F = 3$ to $F = 7$ (21 5°) with $n = 2$, $m = 1$ as in the case of the cone section (dotted line in Fig 2)   The deviations from this at the other two temperatures are similar to those in the case of the cone segments   The devia

tions appear to be systematic, and are also found in a series of measurements in which F_m was determined at fixed I (Crozier, Wolf, and Zerrahn-Wolf (1936–37 a)) They *may* be due to the use of "neutral" filters with slightly different properties in obtaining the low intensities of illumination, the intensities at $F = 3, 4, 5$ were gotten with a 1 10,000 filter, at $F = 6$ a 1 1,000, at $F = 7, 8$ a 1 100, at $F = 9$ a 1 10 filter   The variation data, however (Fig 4), contain no indication of qualitative differences

It is to be noticed (1) that as the temperature is lowered I_m is increased at constant F, and (2) by about the same proportion at all intensities until the maximum F is approached, (3) that the change in I_m is proportionately about the same for both rod and cone sections of the curve, and (4) that the maximum F is independent of temperature and practically independent of intensity   Points (1), (2), and (4) agree precisely with those already established for the flicker response of *Anax*   We have discussed the way in which these phenomena appear to support the "intensity discrimination" conception of recognition of flicker (Crozier, Wolf, and Zerrahn-Wolf (1936–37 c))   We need only call attention here to the fact that the similar shift of the rod and of the cone portion of the curve (Fig 1) is also consistent with this view   Since in equation (2) m (at 21 5°) may be taken to differ for the rod and cone sections, and since the k_2's might not therefore (or in any case) be expected to have the same temperature coefficients, we might not expect that if the theory leading to this equation were applicable change of temperature would lead to the same kind of shift in the positions of the two portions of the entire curve   But if we deal with the operation of a mechanism of discrimination, based upon the involvement of a decay process governing the after effect of a light flash, this is not altogether unexpected   The constancy of F_{max} at different temperatures, and the inconstant curvature of the upper portions of the $F - I_m$ curves, leads one to expect that equation (2) could not apply unless the exponents n and m (apparent orders of reaction in equation (2)) are functions of temperature   Fig 2 shows that the equation used for the curve at 21 5°, with $n = 2, m = 1$ for the cone portion, $n = 2 = m$ for the rods, does not really give a fit for the cones at the other two temperatures   Plotted in terms of equation (1), on a logistic grid (the modulus has been neglected), the exponent p is prac-

tically constant and is independent of temperature (Fig 3)   The *form* of the function is not affected by temperature, its parameters there fore measure a property of the reacting organism, and make possible an investigation of the nature of this property

It is of some interest to consider briefly the difficulties arising if one attempts to estimate temperature characteristics for the underlying processes which might be presumed to be involved in determining the
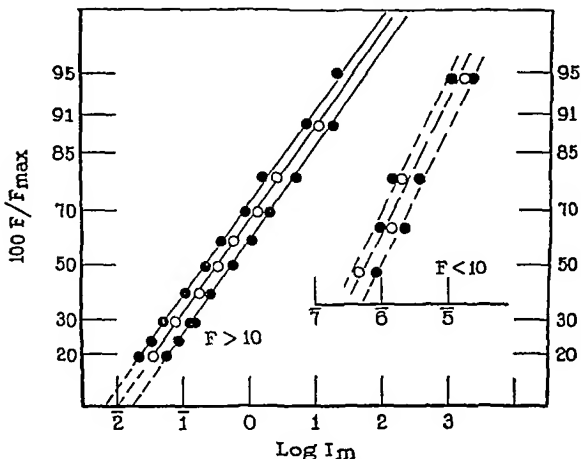

FIG 3 The data of Fig 1 on a logistic grid $F/F_{max} = 1/(1 + e^{-p\log I})$, with $p = 1 37$ for the cone portion $(F > 10)$, $p = 0 5$ for $F < 10$

effects shown in Fig 1   As in the case of *Anax* (Crozier, Wolf, and Zerrahn Wolf (1936–37 c)), the temperature coefficient of intensity at constant F is negative and is a function both of F and of temperature (T) This is scarcely consistent with the idea that a constant amount of photoproduct is involved in the light dark cycle at each F and at constant temperature I_m is a declining and practically rectilinear function of $°C$, the slope constant being a function of F The tem perature coefficient of F ($= 1/2t$) at constant I is positive, and is a

function of I and of T, its values differ for the rod and cone sections, and are not the same as that for I at constant F The conclusion necessarily is that the immediate basis for recognition of flicker is quantitatively a function of F, of I, and of T

IV

The connection between I_m and P E n was discussed in considering the sunfish flicker data at 21 5° (Crozier (1935–36), Crozier, Wolf, and Zerrahn-Wolf (1936–37 a))   It was pointed out that for the lower section of the curve these two quantities are rectilinearly proportional Above a certain intensity the logarithmic plot of I_m vs P E n, however, may be regarded as rectilinear but with a slope (exponent) $= 1$ A slope of 1 was obtained for this upper portion of the curve by plotting log P E n against log $(I + 0\ 10)$ The idea was tentatively entertained that this might signify, for the cone section of the curve, the necessity for a subtraction of the variability due to the continuing participation of rod excitation   This notion must now be somewhat revised   At the other two temperatures used the same type of connection between I_m and P E n is again observed, but with a lateral shift in the position of the graph and a change in its breadth (Fig 4)

The shift of position is such that the lines for 12 4° and for 27 3° are to the right of those for 21 5°   The best fitting central line has, for the lower portion, the equation

$$\text{P E } f_t = I_m(0\ 182) \text{ at } 21\ 5°$$
$$= I_m(0\ 0314) \text{ at } 27\ 3°$$
$$= I_m(0\ 0562) \text{ at } 12\ 4°$$

At given intensity P E n increases in the order 27 3°, 12 4°, 21 5°, while F increases directly as T increases, at given F, P E n goes through a maximum at 21 5°   This is precisely the relationship encountered in the case of the experiments with *Anax* (Crozier, Wolf, and Zerrahn-Wolf (1936–37 c))   If one considers P E f_t as a function of F, curves are obtained of the same general character as for I_m as a function of F (Crozier (1935–36)), but the order of increasing P E n at the three temperatures puts the curves in the sequence 27 3°, 12 4°, 21 5°

These facts substantiate the lawful character of the behavior of P E n as a function of intensity   They are quite inconsistent with any idea to the effect that P E n measures an "error of experiment," the

precision of the mere judgment of the occurrence of the index response depends on the sharpness and clarity of the animal's movements, this increases with increasing I and with elevation of temperature and should lead to a decrease of P E $_{I_1}$, but there is no correlation of this sort
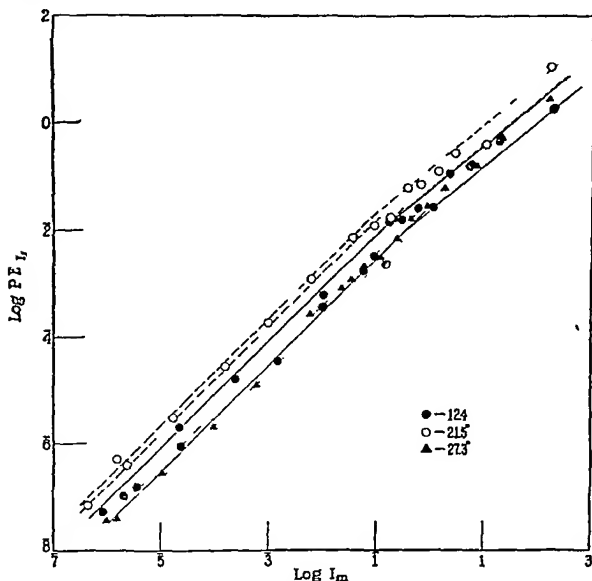

FIG 4 The variability of I_1 for response to flicker log P E $_{I_1}$ vs log I_m See text The slope up to log P.E $_I$ = 2 1 is 1   The vertical breadth of the plot is a function of temperature   The position of the graph is moved laterally as a function of temperature

This may be investigated by a procedure already used in principle for the analysis of variation of geotropic behavior of rats (Crozier and Pincus (1931–32a and b) (1932–33) (1934–35))   In those instances variation of performance was measured not variation of intensity giving threshold performance   The principle

however, is that the relation between variation and mean may be of the same form, although the variation index at a given level of the inciting variable may be changed by altering conditions   On one particular day, for reasons which are obscure, all our fishes used in the present experiments were unusually "restless" and gave quite violent responses to flicker at the thresholds for reaction   The mean intensities obtained on this day, at 12 4°, were

F	$\log I_m$	$P E_{I_1}$
40	0 7340	0 424
50	2 2774	7 146

Reference to Table III shows that these values of I_m each agrees admirably with the results of two other determinations spaced some days apart, and in fact are indistinguishable from the means of these pairs   But $P E_{I_1}$ was 2 5 to 3 8 times as great as in the sets obtained from the same individuals when not in this excited state   The slope of the line connecting $\log P E_{I_1}$ with $\log I_m$, however, agrees precisely with that given by the acceptable measurements   It will be of some importance to investigate the action of general excitatory conditions upon the properties of $P E_{I_1}$, since this may permit a quantitative separation of the rôle of variation due to the reacting mechanism from that due specifically to the mechanism of intensity discrimination   We suggest in another connection (page 430) the importance of a genetic test of the character of the flicker curve which should also give information as to the functional basis of the variability

We have already indicated how the changes in variability appear to be consistent with a theory of response to flicker (Crozier, Wolf, and Zerrahn-Wolf (1936–37 c)), in the case of the *Anax* data, and this need not be repeated here   Further data leading to a more extensive test of the "intensity discrimination" conception will be given by experiments in which the relation of light time to dark time is systematically altered

A noteworthy additional feature of the data has to do with the spread of the band encompassing or describing $P E_{I_1}$ as a function of I_m As found with *Anax*, the "spread coefficient" of $P E_{I_1}$, which may be taken as B in the formula

$$P E_{P E_{I_1}} = B \, P F_{I_1} / \sqrt{N}$$

is also a function of temperature   The scatter of the determinations of $P E_{I_1}$ is greater at the lowest temperature   This may be estimated from $\sqrt{N}(P E''_{I_1} - P F'_{I_1})/P E_{I_1}$ at fixed I_m, where $P E''_{I_1}$ is the upper limit of the band, $P E'_{I_1}$ the lower limit   Approximate values for this fraction are 12 3° *3 32*, 21 5°, *0 906*, 27 4°, *1 77*, for the lower

section of the graphs in Fig 4, for the upper section the proportionate spread is *2 66, 3 05, 1 84* These values are better established at 12 3° and at 27 4° than at 21 5°, and the latter series was made with a different series of individuals, a larger number of determinations in the rod region at 21 5° would undoubtedly give a wider spread The
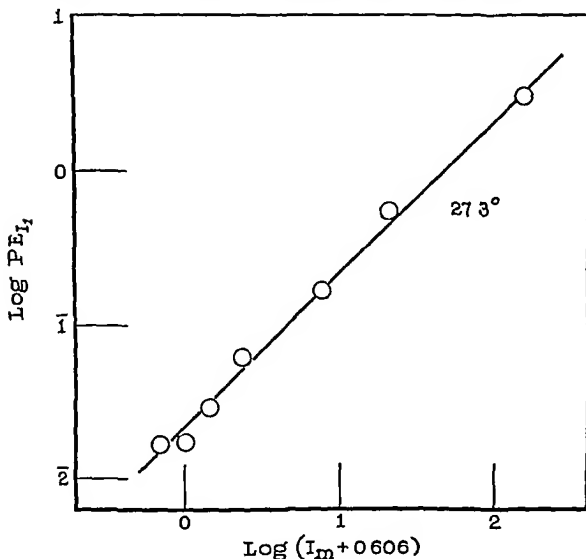

FIG 5 Log P E $_{I_l}$ vs log (I_m + c), for values of P E $_{I_l}$ > antilog 2 1, to give a slope = 1

general implication, as in the *Anax* case, is to the effect that the spread coefficient for P E $_{I_l}$ is less the higher the temperature, corresponding to an increase in the speed (hence "precision") of the processes leading to reaction when the excitation is adequate

The change of slope of the logarithmic graph of P E $_{I_l}$ against I_m occurs at a higher value of I_m in the order 21 5°, 12 4°, 27 3°, but the

level of P E $_{r_1}$ at which the transition is apparent is substantially the same (antilog $\bar{3}$ 9)   The upper portions of the bands can be described as straight, with slope $= 0$ 83 (Fig 4), or, by addition of a constant to I_m, as rectilinear with a slope of 1 (Fig 5)   Adding a constant to I_m is equivalent to subtracting a constant (0 00745) from P E $_{r_1}$ We have suggested (Crozier, Wolf, and Zerrahn-Wolf (1936–37 c)) that this might signify the correction for "full excitation of rods"   The values of log I_m at which the correction for this purpose becomes necessary are approximately 12 4°, $\bar{1}$ 15, 21 5°, $\bar{2}$ 5, $\bar{2}$7 3°, $\bar{1}$ 5   These levels show no exact correspondence with the order of positions of the transitions to the cone portions of the flicker curves (Fig 1), the out-of-place value is that at 27 3°   If we suppose, however, that the approximately constant level of log P E $_{r_1} = \bar{3}$ 85 corresponds to the full effect of excitation of rods, we can perhaps believe that with lower absolute values of P E $_{r_1}$ at given I_m, this contribution to the total variation should be more effective   It is possibly not without sig- nificance that the two determinations at $F = 25$ (27 3°) and the two at $F = 15$ (12 4°) show a wide difference at precisely the level (Fig 4) which is assumed to mark transition to the lower slope

<div align="center">v</div>

A special problem arises in dealing with the flicker measurements with eyes of vertebrates   It has to do with the separate functioning of rods and of cones (Hecht and Verrijp (1932–33b), Hecht, Shlaer, and Smith (1935)), reflected in the two segments of the curves of Fig 1 This double type of curve is not obtained with animals in which there is no evidence of duplex constitution of the excitable retinal fields, such as *Anax* and the bee (Crozier, Wolf, and Zerrahn-Wolf (1936– 37 b), Wolf (1933–34))   The immediate question is, From the curves in Fig 1 is it possible to deduce properties of the mechanism of excita- tion?   A practical aspect of this concerns the precise mode of super- position of the cone effect upon that assignable to the rods   Two extreme positions are possible   either the response to flicker is deter- mined by the most sensitive excitable elements, or it is due to a critical value of the summated excitation of all the elements excitable under the conditions   For diverse visual functions one or the other view might be necessary   And a still different theoretical position is

possible—if not indeed required   The point has been made that it is inadmissible to deduce a theory of mechanism from measurements of average critical excitation energies alone, since, in view of the variation of performance inherent in biological systems, an essentially arbitrary procedure must be adopted if theory is to be tested by curve fitting (Crozier (1935–36))   The functional connection between flicker frequency and fluctuation of intensity to give the end point is conditioned by the fluctuating effect produced by a given intensity To assume that this fluctuation is governed by peripheral events, in the eye, is arbitrary and unnecessary   We must deal solely with "effects," defined in the only manner in which they can be defined, namely in terms of the result by which we recognize them   We have dealt with marginal response to flicker in terms of intensity discrimination, considering that a discrimination must be possible between the effects of flashes of light and the effects of their after action (after image) (Crozier (1935–36))   This view is required by the relation of flicker recognition curves to temperature, and it accounts satisfactorily for the variability functions (Crozier, Wolf, and Zerrahn Wolf (1936–37 c))   The total direct effect of a light flash within a fixed time interval must be the result of the total of nerve impulses to which it gives rise   There is enough general evidence to show that at least two important elements are to be recognized as taking part in this total the number of active retinal elements, and the number of impulses proceeding from each (cf Hecht (1934))   The particular aspect of performance selected for measurement will determine whether (1) number of active elements, governed by the relation between threshold distribution and intensity, or (2) total density of nervous effect, is the limiting variable   The intensity discrimination view of marginal response to flicker calls for a form of the integrated density of effect idea the total effect of a flash is just discriminated from the total effect in the interval after the flash   It must be presumed that different nervous elements of any one type (e g , cones) will vary, at a given time, in the magnitude of the direct effect produced in them by the light flash and in the associated after effect, and consequently in their ability to participate in the activity of discrimination   The phrase "nervous element" is used deliberately, to include peripheral sense cell *and* its central nervous representation, the ultimate decision

as to the effective locus, very possibly central, is not prejudiced by this terminology, and the problems of detail are kept more fairly in view   It is to be conceived that the variation of capacity to partici-pate in the discrimination of flicker, under constant external condi-tions, is analogous to the change in this capacity produced by altering the temperature   At constant temperature, with increasing I and F, an intensity is ultimately reached beyond which, at still higher intensi-ties, a *longer* time must be allowed for the decay of after effect in the dark interval if the effects in the light and dark intervals are to be just discriminated   This is not to be confounded with the require-ments of minimum time for threshold excitation of a sensory cell Diagrammatically, this may be represented in a manner similar to that used in our preceding paper, in which the action of temperature was considered (Crozier, Wolf, and Zerrahn-Wolf (1936–37 c))   With sufficiently high intensity of flash little change in the decay curve of the after effect is to be produced by further increase in intensity, so that comparison of the areas under the light flash curve and the decay curve, using the criterion of marginal recognition of difference, will require longer time intervals for increases of intensity beyond this point, this means that for a given F, beyond the maximum, as I in-creases a smaller and smaller number of excitable elements will be able to contribute to the recognition of flicker

Precisely this finding has been made at the upper end of the flicker curve for man, with small test fields (Hecht and Verrijp 1932–33 a)), the curve bends, and the function is no longer monotonic   With bee, *Anax*, and sunfish this would in all probability be found if, with our apparatus, sufficiently precise measurements could be made with much higher intensities than we are able to use   The drop in the cone curve is pushed to higher intensities when a flickered area is surrounded by a non-flickering field (Hecht, Shlaer, and Smith (1935)), when the whole eye is exposed to flicker the failure of rods to be involved at high intensities may in effect provide a kind of steady surround at the periphery of the retina, this may result in the absence of a drop in the curve for the whole eye until very high intensities are reached   Under proper conditions its counterpart might well be detectable in connec-tion with the rod curve   Certain human flicker curves, but not all, do show a significant depression at or slightly before the onset of the

cone rise (Ives (1912), Hecht and Verrijp (1932–33 a)), which tends
to be more extensive in the case of retinal areas further toward the
periphery and (data of Lythgoe and Tansley (1929)) with more com
plete light adaptation   In the data on the sunfish (Fig 1) there is no
dip, but a distinct and significant upward trend starting at about log
$I_m = \overline{4}\,0$ or above   This is shown in all four independent sets of
measurements (cf also Crozier, Wolf, and Zerrahn Wolf (1936–37 a))
The beginning of this rise is at progressively higher intensity as the
temperature falls (Fig 1)   It provides anchor points by which to
judge the results of an attempt at downward extrapolation of the cone
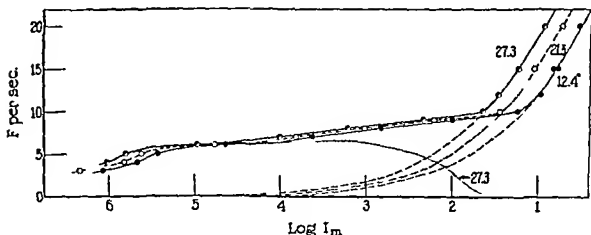
Fig 6 The result of extrapolating the logistic curves for the cone portions of
the flicker response curves (Fig 3)   The extrapolation cuts the log I_m axis at each
temperature at a point appropriate to account for the rise near log $I_m = \overline{4}\,0$ in
Fig 1   The difference curve (dotted) is discussed in the text (only that for 27 3°
is shown)

portion of the curve   Any such extrapolation is somewhat dubious at
best, its outcome is to be evaluated by the usefulness and coherence
of its predictions   The rectilinear character of the cone curve on a
logistic grid (Fig 3), down to $F = 10$, invites backward extrapolation
to lower flicker frequencies by means of this relationship   The extent
of the extrapolation is only 20 per cent of the total range of F The
outcome, shown in Fig 6, indicates that the extrapolated origin of the
cone curve comes at a value of I_m which at each temperature coincides
with the location of the "bump" on the original $F - \log I_m$ curve
The logistic plot of F_m as a function of I at 21 5° (data in Crozier,
Wolf, and Zerrahn Wolf (1936–37 a)) agrees precisely with that for

I_m at this temperature   Accepting this extrapolation we may then determine by difference the contribution made by rod excitation at flicker frequencies $< F = 10$ to the total effect upon which response to flicker is based   Above $F = 6\,3$, a level apparently not influenced by temperature (just as with the cone maximum), this contribution declines   The decline is associated with higher values of I_m the lower the temperature   This is consistent with our view that as the temperature is lowered the velocity constant for after effect decay is less, as then at a given F a higher intensity should be permissible if a given kind of difference is to be obtained between effect of light flash and its decay   We therefore regard the results of this analysis as consistent with the indications provided by the human flicker data They lead to the view that in the region of intensities where both rods and cones function to contribute to the effect determining response to flicker the contributions of the two are additive, but that the rod contribution fades out of the picture as higher and higher intensities (and therefore higher flicker frequencies for the cone curve) are used On this basis also the photostationary state character of the shape of the flicker curve is clearly illusory

This mode of separation of rod and cone curves involves arithmetic addition of flicker frequencies in the region of overlapping, a procedure which raises a number of questions   The logistic formulation of the cone curve implies that critical flicker frequency measures an additive property of some feature of excitation which is governed by log I Why it should be so is another matter   A suggestion is given by data on critical flicker frequency as a function of area of retina   The determinations of the flicker curve for equal areas at various locations on the human retina show that F increases as the total number of active elements involved at the given intensity becomes greater (Hecht and Verrijp (1932–33 a))   The relation between F and wave length is of the same kind (Hecht, Shlaer, and Smith (1935))   At a constant I, F increases as the area is made greater at a given location (Granit and Harper (1930))   For the range of intensity where F is very nearly proportional to log I, it is nearly proportional to log *area* at fixed I If increase of area at fixed I means increase in number of elements of effect, and increase of I with constant area has the same sort of significance, then the relationship between them (at least over

this range) is expressible as a simple power function   In equation (1) we can then write

$$F = F_{max} /(1 + e^{-h \, log \, cN})$$

where h and c are constants and N signifies the variable by means of which the number of active elements of the effect determining F is increased   For N may be inserted either *area* (A) or *intensity* (I), on the basis that $A \equiv q \, I^{g}$ Essentially this situation has been pointed out by Hecht altering the area of an excited field merely shifts the position of the log F *vs* log I curve without changing its shape (Hecht, Shlaer, and Smith (1935))   Wald[1] has been able to show that the dependence of various visual functions upon area is quite satisfactorily accounted for on the assumption of a population distribution of excitabilities as a function of log I Accepting this, F corresponds simply to number of excitation elements entering into the determination of critical flicker   Its additive character in the case of contributions from rods and from cones is therefore not mysterious   Since F has the dimensions of a frequency, we must consider that the speed of the process as a whole, due to the totality of excitation given by equal light and dark intervals ($i \, e$, its central nervous representation), which achieves reaction to flicker depends upon and is directly proportional to the number of excitation elements (units of excitation) involved   This is a reasonable result despite the complexity of the comparisons apparently concerned in the intensity discrimination which we have conceived to be basic to the response to flicker   It is consistent with the relationship of F to temperature at fixed I, F increases as T increases

In another way the additive property of critical flicker frequency is also shown by its behavior at various retinal locations during the progress of dark adaptation, in the data of Lythgoe and Tansley (1929)   The evidence there given indicates addition of the effects respectively due to rods and cones as these are changing during adaptation in the fovea, F at given intensity falls as dark adaptation proceeds, with declining rate of fall, while at the periphery F rises

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[1] We are indebted to Dr George Wald for the opportunity to consider his results in advance of their publication

during dark adaptation, at intermediate locations it goes through a minimum, an effect not detected in a night-blind subject

The conception here arrived at, of the essential proportionality of critical F to magnitude of sensory effect, has certain important consequences when combined with the intensity discrimination theory of response to flicker   These we shall attempt to utilize in a later paper

If this mode of dissection of the rod and cone contributions to the flicker response curve is correct in principle, then it will be observed that there is provided a case in which it might be argued by some that the coming into action of retinal cones progressively "inhibits" the effective performance of rods—one type of "retinal interaction" But the argument would be pointless because (1) the cone curve, at sufficiently high intensities, also bends over, and (2) the effect is less obscurely accounted for by consideration of the basis of recognition of flicker

There are two rather different tests which can be made of predictions called for by our view   (1) The flicker curve for a cone-free human subject should be found to rise to a low maximum and then to decline (2) It should be possible to find fishes in which the separation of the rod and cone curves is in one way or another more pronounced, so that several forms of transition to the cone section of the flicker curve could be measured   The data on several human subjects (Hecht and Verrijp (1932–33 a)) suggest individual differences   Fresh water teleosts have the advantage for this purpose that hybridization is possible with suitably selected material   A genetic test of the basis for differences in flicker curves could then be made in the manner employed for the analysis of geotropic response (Crozier and Pincus (1931–32 b))   It is to be expected also that by this procedure the capacity to exhibit variation in critical intensity for response to flicker may be submitted to functional dissection, since this must be in part determined, as to absolute amount, by properties of the reaction system of the species and in further part by the active mechanism of discrimination of flicker

VI

SUMMARY

The curve connecting mean critical illumination (I_m) and flicker frequency (F) for response of the sunfish *Lepomis* (*Lnneacanthus*

gloriosus) to flicker is systematically displaced toward lower intensities by raising the temperature   The rod and cone portions of the curve are affected in a similar way, so that (until maximum F is approached) the shift is a nearly constant fraction of I_m for a given change of temperature   These relationships are precisely similar to those found in the larvae of the dragonfly *Anax*   The modifications of the variability functions are also completely analogous   The effects found are consistent with the view that response to flicker is basically a matter of discrimination between effect of flashes of light and their after effects,—a form of intensity discrimination   They are not consistent with the stationary state formulation of the shape of the flicker curve

An examination of the relationships between the cone portion and the rod portion of the curves for the sunfish suggests a basis for their separation, and provides an explanation for certain "anomalous" features of human flicker curves   It is pointed out how tests of this matter will be made

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THE POTENTIAL AND RESPIRATION OF FROG SKIN

I The Effect of the Homologous Carbamates   II The Effect
of Certain Lysins

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INTRODUCTION

The subject of the relation between metabolism and the sponta-
neously occurring potential differences observed in certain tissues has
been extensively investigated by means of experiments which fall into
two general categories   The first type of experiment consists in
showing that regions which have a higher metabolic rate, either
because they are actively growing, or because their O_2 consumption or
CO_2 output is greater, or because they reduce methylene blue more
quickly, are positive to regions where the metabolism is lower   This
has been done for stems of *Obelia* and for several varieties of growing
root tip (Lund (1925), Lund and Kenyon (1927)), and Lund interprets
the potential difference in general as due to "the flux equilibrium of the
oxidation reduction systems" in the cells, the experiments, however,
are inconclusive in that differences in metabolism or in oxidation-
reduction potential are not the only factors which can give rise to the
potential differences   The second type of experiment seeks to show
that the O_2 consumption of a tissue in which spontaneously occurring
potential differences can be measured is depressed by O_2 lack or by the
addition of various substances in the same way as are the observed
potential differences   More specifically, Lund (1928 *a*) has shown that
KCN depresses the O_2 consumption and the potential difference to the
same extent in the case of frog skin, and Francis (1934) agrees with the
conclusion in a general sort of way   The results of experiments on the
effect of varying the O_2 tension to which the skin is exposed are very
conflicting, probably because of the different methods used   Lund
(1928 *b*) finds both O_2 consumption and potential to be reduced when

the O_2 tension is diminished, although not to the same extent, indeed, he says that the same increase in O_2 tension may stimulate respiration as much as 1600 per cent and yet increase potential by 100 per cent only　His method for measuring O_2 concentration and consumption (the Winkler method for dissolved O_2) is open to criticism, however, and both Adolph (1929) and Francis (1934) find a different relation between O_2 tension and O_2 consumption from that described by Lund Francis (1934) concludes that the potential and respiration depend in the same way on the O_2 concentration, but Taylor (1935) says that the lowering of potential is usually about 20 per cent greater than the lowering in O_2 consumption when the skin is exposed to N_2/O_2 or CO/O_2 mixtures, finally, there is disagreement as to the effect of high concentrations of O_2 on the respiration and potential (Lund (1928 b), Francis (1934), Taylor (1935))

No quantitative work has yet been done on the effect of narcotics on potential and O_2 consumption when these are measured simultaneously, although Boell and Taylor (1933a) have investigated the effect of the homologous carbamates on frog skin potential and shown that they depress it, the purpose of the first part of this paper is accordingly to show that the effect of the carbamates on frog skin potential is not closely related to the effect on O_2 consumption　In the second part of the paper it will be shown that several lysins, which have certain properties in common with the carbamates, abolish the skin potential without reducing the respiration

I　The Effect of the Homologous Carbamates
Materials and Methods

1　Frog Skin　—The frogs (*Rana pipiens*) were used during the winter months (December–March), kept in the cold, and acclimatized at 20°C for 72 hours before the skin was removed　For the measurement of respiration, pieces of skin about 200 mg in weight were taken from the ventral surface, the dorsal surface, and from the upper part of the legs, but for the potential measurements only skin from the ventral surface and from the legs was used, as dorsal skin usually gives small potentials　In all cases the measurements of O_2 consumption and of potential were begun within a few minutes after the skin was removed

2　Respirometry　—The measurements of O_2 consumption were made at 25°C in Fenn respirometers with side cups from which the carbamates, etc, could be added by tipping the instrument　One piece of skin was used in each respirometer cup, which also contained 0 5 cc of frog Ringer (without carbonate), and

1 cc of the carbamate solution to be added was placed in the corresponding side cup After 30 minutes for equilibration, the normal O_2 consumption was measured over a period of 30 minutes, this normal O_2 consumption remains remarkably constant for a period of at least 2 hours

It is convenient here to give the values for O_2 consumption which we have obtained These can be given either in terms of the wet weight of the skin (mm ^{3}/gm /hr), or in terms of dry weight (Q_{O_2}) [1] and vary with the part of the body from which the skin is taken The results of some 400 determinations are shown in Table I

These values are about the same as those obtained by Francis (1934), who finds a Q_{O_2} of $-1 08$, but lower than those of Taylor (1935), who gives 330 mm ^{3}/gm /hr for wet skins and higher than those of Adolph (1929) and of Lund and Moorman (1931) whose values for wet skins are 125 mm ^{3}/gm /hr and 139 mm ^{3}/gm /hr respectively The only investigators who seem to have noticed that the O_2 consumption depends on the region from which the skin is taken are Williams and Sheard (1932)

TABLE I

Location	Wet skin, mm³./gm /hr	Dry skin Q_{O_2}
Dorsal	185	$-0 87$
Ventral	236	$-0 97$
Leg	265	$-1 15$

After the normal O_2 consumption has been observed for 30 minutes, the apparatus is tilted and the narcotic thus added to the skin in the respirometer vessel Readings are made each 10 minutes for another 30 minutes, at the end of which time a new and steady rate of O_2 consumption is always attained, the effect of the narcotic on the respiration being complete within 20 minutes irrespective of the narcotic concentration The rate of O_2 consumption at the end of this time is then expressed as a percentage of the normal rate and, to allow for small variations in the effect the results of eight experiments are averaged for each concentration of narcotic The extent to which any given concentration of carbamate depresses the skin respiration may vary by ±10 per cent, and the average depression of respiration observed in eight experiments with one narcotic concentration varies by ±3 per cent at most

3 Potential Measurements —These were made by the use of very simple apparatus The skin is stretched over the end of a small glass tube, and secured in

[1] Q_{O_2} is defined as mm ^{3} O_2 consumed per hr per mg of dry tissue The O_2 consumption in mm ^{3}/gm /hr for wet skin, when divided by 213, gives the Q_{O_2} with great exactness

position with rubber bands, the tube is then inverted, so that the skin dips below the surface of Ringer's solution contained in a small flat receptacle   A small amount of Ringer is placed in the tube, so as to bathe the inner surface of the skin and equalize the hydrostatic pressure, and calomel half-cells, prepared with 0 6 per cent NaCl, dip into the fluid in the tube and in the receptacle respectively We have found agar-silver-silver chloride electrodes very unsatisfactory   The potentials are measured in the usual way, with a high resistance galvanometer as a null instrument, and at a temperature of 25 ±1°C

The skin potentials are observed at 15 minute intervals for periods from 1 to 3 hours, and only if a steady potential is finally attained is the experiment continued   The final potential attained is very variable from frog to frog, and, as Williams and Sheard (1932), Boell and Taylor (1933a), and Francis and Pumphrey (1933) have observed, the ventral skin gives consistently higher potentials than the dorsal skin   The skin of the leg usually gives a potential between the two   In view of this variability, we have made it a rule to use only skins which give a potential of between 25 and 40 mv at equilibrium[2] There seems to be no relation between the O_2 consumption of the skin and its potential at equilibrium, as follows from the fact that the former is very constant, whereas the latter is exceedingly variable

When a steady potential is attained, the Ringer's solution on both sides of the skin is removed and replaced by a solution of the narcotic in Ringer   The change in potential is followed, and at the end of 30 minutes a new and steady, or sometimes slowly falling, potential is reached   In order that the determination may be comparable to the determination of the effect of the narcotic on the O_2 consumption, the potential at the end of 30 minutes is taken as the final one, and is expressed as a percentage of the equilibrium potential before the addition of the narcotic   The variability in the effect of the carbamates on skin potential is much greater than the variability in their effect on respiration, and so the results have to be based on the average of a number of experiments, and a standard error attached   The number of individual experiments required for the determination of the effect of a given concentration of carbamate depends, in fact, on the magnitude of the effect itself, thus, it is easy to show that there is no diminution in potential or that the potential is completely abolished, but

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[2] Boell and Taylor (1933a) appear to be satisfied that the effect of a carbamate on frog skin potential is independent of the original potential difference observed across the skin, provided the effect is expressed as a percentage of the original equilibrium potential, even if this varies from 5 to 75 mv   This has not been our experience, for skins with a low initial potential are frequently affected by the addition of a carbamate to a greater extent than are skins with high initial potentials   The percentage effect of a carbamate, however, seems to be relatively independent of the initial potential when the latter is between 25 and 40 mv The same kind of difficulty does not arise with respect to the O_2 consumption, for this is subject to much less variation

a large number of individual experiments are required when the reduction in potential is only partial

RESULTS

The results obtained with four carbamates, ethyl, isopropyl, n butyl, and isoamyl, are shown in Table II [3] The first column gives the molar concentration of the narcotic, the second the O_2 consumption 30 minutes after the application of the carbamate, expressed as a percentage of the normal O_2 consumption, and the third the potential

TABLE II

Carbamate	Concentration	O_2 consumption	Potential
	M	*per cent*	*per cent*
Ethyl	0 2	100	50
	0 3	67	20
	0 4	50	0
	0 8	20	0
	1 5	18	0
Propyl	0 04	100	100
	0 07	58	70
	0 10	35	37
	0 15	20	2
	0 19	19	0
Butyl	0 02	80	100
	0 04	36	80
	0 06	20	37
	0 08	17	10
	0 10	20	0
Amyl	0 006	80	90
	0 01	48	41
	0 0125	34	23
	0 018	19	0

attained 30 minutes after the addition of the carbamate, also expressed as a percentage of the normal equilibrium potential of the skin   In all cases the concentration of narcotic is that actually present in the fluid bathing the skin in the respirometer vessel, for the 1 cc of narcotic

[3] All the carbamates used were obtained from the Eastman Kodak Co   In some of the earlier experiments we found that ethyl carbamate obtained from another source had less narcotic effect   Boell and Taylor use the words car bamate' and "urethane synonymously

added from the side cup is diluted with the 0 5 cc of Ringer in the vessel containing the skin

These results are shown graphically in Figs 1–4, the O_2 consumption being shown by the line passing through crosses and marked A, and
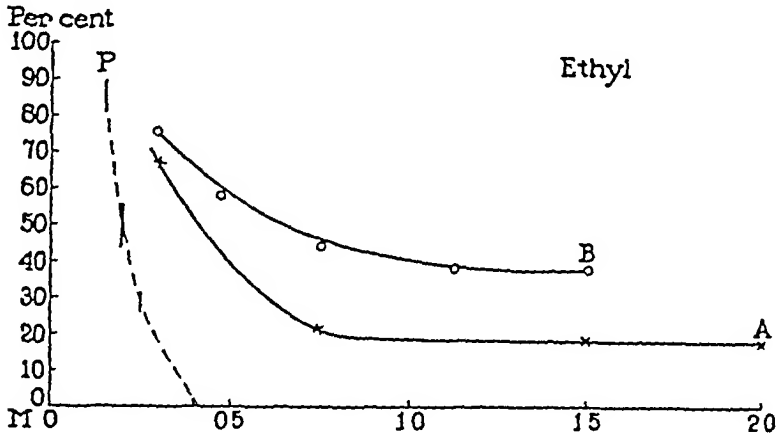

FIG 1 Ethyl carbamate   Curve P, effect on potential, curve A, effect on O_2 consumption   (Curve B shows results obtained in the experiments on adsorption of the carbamate, see text for explanation) Ordinate, potential or O_2 consumption as a percentage of initial potential or O_2 consumption, abscissa, concentration of carbamate in the system
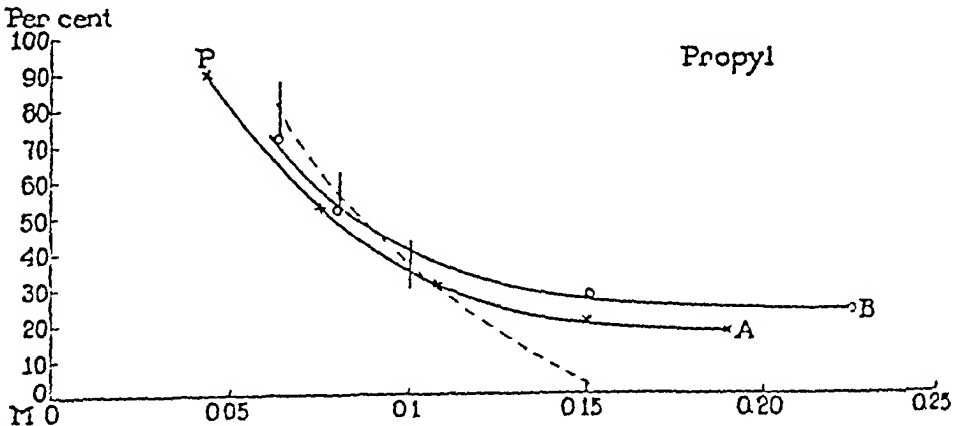
FIG 2 Propyl carbamate   Ordinate and abscissa, etc , as in Fig 1

the potentials by the dotted line marked P   In the case of the curve for the potentials, the line is drawn through the means for a variable number of experiments with each narcotic concentration, and the vertical lines show the size of the standard errors

Speaking generally, the curve for the reduction of potential by increasing quantities of narcotic pursues a course different from that of the curve for the reduction of O_2 consumption In the case of propyl, butyl, and amyl carbamates, the respiration is first reduced in a concentration which does not affect the potential, and the potential is abolished in a concentration which reduces the O_2 consumption to
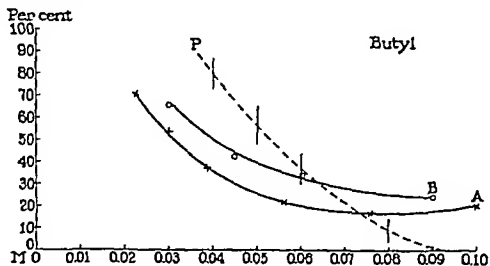
FIG 3 Butyl carbamate Ordinate and abscissa, etc , as in Fig 1
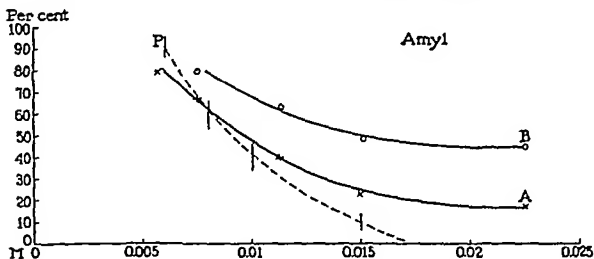
FIG 4 Amyl carbamate. Ordinate and abscissa, etc , as in Fig 1

about 20 per cent, a point beyond which it does not seem possible to reduce it further [4] The curve for the reduction of the potential thus

[4] In the case of all the carbamates the curve relating residual respiration to concentration shows a tendency to rise when the carbamate concentration is very great Thus the O_2 consumption is reduced to 18 per cent by the addition of 1 5 M ethyl carbamate, but only to 26 per cent by the addition of 2 25 M This point is not shown in the figures

crosses the curve for the reduction of the O_2 consumption. The difference between the two curves is very marked in the case of ethyl carbamate where the potential is reduced to zero in a concentration which reduces the respiration to 50 per cent only.'

Traube's Rule and the Effective Concentration of the Carbamates

The data obtained in the foregoing experiments can be used to test the validity of Traube's rule for members of a homologous series, this rule stating in its simplest form that the ratio between the isoactive concentrations of any two adjacent members of a homologous series is 3 1. Boell and Taylor (1933a) conclude that the carbamates conform to Traube's rule in depressing the potential of frog skin, and

TABLE III

Carbamate	50 per cent potential		50 per cent respiration	
	Concentration	Ratio	Concentration	Ratio
	M		M	
Ethyl	0 2	2 4	0 4	5 2
Propyl	0 05	1 7	0 078	2 4
Butyl	0 070	5 6	0 032	3 3
Amyl	0 009		0 0096	

the concentration of narcotic actually present in the regions of the cells affected than to the concentration added to the system as a whole The quantity of narcotic "adsorbed" by the skin at the end of any given time from a solution of any given concentration of carbamate can be found in the following way

A piece of frog skin of the size ordinarily used in obtaining the curves marked A in Figs 1–4 (about 200 mg in weight) is placed in 1 5 cc of a selected concentration c_1 of carbamate contained in a respirometer vessel which is rocked to and fro for 30 minutes at 25 C The respiration is not recorded the only purpose of the respirometer being to duplicate the conditions under which the standard curves marked A were obtained At the end of 30 minutes 1 cc of the fluid surrounding the skin is transferred to the side cup of another respirometer containing a fresh piece of skin in 0 5 cc of Ringer, the normal respiration of this piece of skin is recorded for 30 minutes and then the fluid from the side cup is tipped into the respirometer vessel The residual O_2 consumption at the end of another 30 minutes is found and expressed as a percentage of the normal O_2 consumption of the skin, already measured Since a relation exists between the residual respiration at the end of 30 minutes and the concentration of a carbamate this relation being shown in the curves marked A the concentration c_2 of the carbamate added from the side cup can be read off from the standard curves The difference $\Delta = (c_1 - c_2)$ obviously gives the quantity of carbamate adsorbed by the first piece of skin at the end of 30 minutes

The results obtained for various concentrations of different carbamates are shown in the curves marked B in Figs 1–4, these showing the relation between the residual respiration of the second piece of skin and c_1, the concentration of carbamate added to the first piece of skin Each point is an average of six experiments, and the values found show a variation not exceeding ± 5 per cent Table IV shows the relation between Δ, the quantity of carbamate adsorbed by the first piece of skin, and c_2, the quantity left free, both Δ and c_2 being given in molar concentrations To convert them to milligrams of carbamate adsorbed or left free in the experimental systems, it is only necessary to multiply the figures in the table by 1 5 times the molecular weight of the carbamate

When log Δ is plotted against log c_2, a good straight line results for each carbamate, the quantity of carbamate taken up by the skin is accordingly related to the amount left free by the expression

$$\Delta = A c_2{}^y$$

which is the same as that for the adsorption isotherm. The slope of the lines, which determines the constant n, is substantially the same for each of the carbamates, but the numerical value of n is exceptionally small (0 2 approximately), this makes one reluctant to accept the

TABLE IV

Carbamate	c_i	Δ
Ethyl	0 27	0 03
	0 36	0 14
	0 46	0 29
	0 52	0 58
Propyl	0 056	0 0065
	0 080	0 0080
	0 115	0 0350
	0 125	0 10000
Butyl	0 024	0 0060
	0 035	0 0100
	0 042	0 0180
	0 050	0 0400
Amyl	0 0060	0 0015
	0 0078	0 0032
	0 0090	0 0060
	0 0105	0 0120

linear relation of $\log \Delta$ and $\log c_i$ as evidence of the carbamates being concentrated in the neighborhood of the skin by an adsorption process alone [6]

The quantities of the carbamates taken up by the skin when the O_2 consumption is reduced to 50 per cent, or to 20 per cent, do not conform to Traube's rule any better than do the quantities which are present in the system as a whole The quantities of adsorbed carbamates which correspond to a 50 per cent reduction in O_2 consumption,
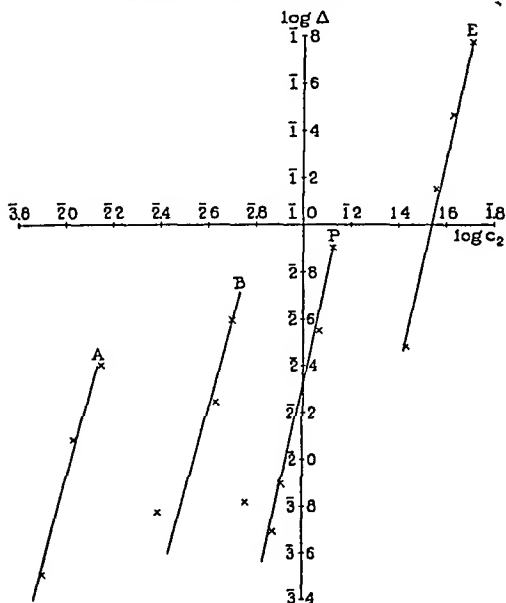
FIG 5 Adsorption of the carbamates Curve E ethyl P, propyl, B, butyl and A, amyl Log Δ plotted against log c_2

for example, are in the ratios 1 8 7 11 6 58 for the successive homologues, instead of in the ratios 1 3 9 27, as Traube's rule requires

II The Effect of Certain Lysins

The effect of the carbamates on the potential and O_2 consumption of frog skin shows that these two properties are not affected identically,

but does not supply evidence that they can be affected independently We have therefore studied the effect of another class of surface active substances, viz the simple lysins particularly as Boell and Taylor's work (1933 a, 1933 b) suggests a relation between the effect of the carbamates on skin potential and their surface activity The lysins which we have used are saponin, sodium taurocholate, and sodium glycocholate, and the methods employed are identical with those described above

Quite unlike the carbamates, these simple lysins in proper concentration completely abolish the skin potential, but leave the O_2 consumption either unaltered or slightly increased The first column of Table V gives the dilution of the lysin, the second the O_2 consumption 30 minutes after the addition of the substance, and the third the poten-

TABLE V

Lysin	Dilution 1 in	O consumption	Potential
		per cent	*per cent*
Saponin	50	100	0
	100	140	15
	500	100	50
Taurocholate	100	100	0
Glycocholate	100	110	0
	200	135	20

returns to its normal rate and continues at this rate for hours, whereas the skin potential is entirely abolished

DISCUSSION

In interpreting these results, it must be borne in mind that frog skin is a heterogeneous tissue, and that the potential differences may depend on properties which are spatially defined, for example, they may arise in the neighborhood of the skin surfaces only   When one measures the O_2 consumption of the whole skin, one is not necessarily measuring the O_2 consumption in the regions which determine the potential difference, and so it is not remarkable that substances such as the carbamates or the simple lysins should affect the O_2 consumption and the potential differently, particularly as the substances themselves are in all probability heterogeneously distributed   To take an extreme case in the lysins which abolish potential but leave respiration un changed, these substances may be concentrated in the neighborhood of the skin surfaces, and while these may be regions responsible for the maintenance of potential, their contribution to the total O_2 consump tion of the skin may be very small   Even if one were to admit that the potential difference observed across a tissue had its origin in metabolic processes of which O_2 consumption was a measure, it would be impossible, strictly speaking, to determine the nature of the relation except in a tissue in which there was no spatial separation of the seat of the potential and of the respiration respectively

Bearing this in mind, the results can be considered in relation to two theories regarding the origin of the potential difference across frog skin   The first is the general theory most clearly expressed by Francis (1934), which requires two things only (a) a respiratory process affording a constant source of diffusible ions, and (b) a partial separa tion of oppositely charged ions, which may result from there being a concentration gradient, a distribution of dissolved salts between two

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the apparent increase in the O_2 consumption   There are two reasons why we think that it may not   The first is that there is a very poor relation between the pH of the added lysin and the apparent increase in O_2 consumption, and the second is that the apparent increase sometimes lasts as long as 2 hours   There may thus be a real stimulating effect on the O_2 consumption, such as undoubtedly occurs in the case of ethyl carbamate

immiscible solvents, or the presence of a semipermeable membrane, the integrity of which may also depend on metabolic processes   There are a sufficient number of possible ways in which a narcotic may act in such a system to account for all the experimental results, the effect of a carbamate, for instance, need not be such as to depress potential and respiration equally, and in an extreme case a lysin might destroy the integrity of a semipermeable membrane or other barrier to equal ionic diffusion without affecting the metabolic process which gives rise to the diffusible ions, and which is measured in terms of the O_2 consumption

The second theory is that of Lund (evidence for which is well summarized in Lund (1930)), which accounts for the potential difference in terms of the "flux equilibrium potential of oxidation-reduction systems" in the cells   The supporters of this theory would have little difficulty in accounting for the results contained in this paper in so far as they concern the unequal effect on O_2 consumption and potential difference produced by the carbamates, for they have used the conception of "flux equilibrium" to account for the similar disparity in effect on O_2 consumption and potential observed with reduced tensions of O_2 (see Lund (1928 b))   The abolition of the potential by the lysins, without there being any reduction in O_2 consumption, cannot be explained in this way, but even this result does not disprove Lund's hypothesis if the frog skin is recognized as a heterogeneous tissue, for it can be argued that the potential differences are due to oxidation-reduction processes in spatially delimited regions of the skin, these being affected by the lysins, but not contributing appreciably to the total O_2 consumption of the bulk of the skin   The idea of heterogeneity, however, is essential, and it ought to be remarked that a theory such as Lund's, which postulates a specific relation between O_2 consumption and potential difference and yet is sufficiently elastic not to be disproved by a type of experiment which shows that the variables can be affected independently (the foregoing), cannot derive substantial support from similar experiments (those involving variations in O_2 tension) which show that, under other circumstances, the variables are affected in a like manner

SUMMARY

Measurements of the O_2 consumption and of the potential of frog skin, made under comparable conditions, show that the homologous carbamates (ethyl, propyl, butyl, and amyl) reduce both the O_2 consumption and the potential, but not in a similar manner In this respect, the effect of the carbamates is like the effect of reduction in O_2 tension The simple lysins (saponin and the bile salts), on the other hand, abolish the potential without reducing the O_2 consumption at all

Irrespective of whether one considers the concentration of carbamate in the entire system or the amount of carbamate adsorbed by the frog skin, Traube's rule relating the effect of a carbamate to its position in the homologous series does not seem to apply

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THE RESPIRATORY QUOTIENT OF SEEDLINGS OF LUPINUS ALBUS DURING THE EARLY STAGES OF GERMINATION

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I

In the ungerminated seeds of *Lupinus albus* there is a fat oxidation system which becomes active on the addition of a sufficient quantity of water (Craig (1936)) The question arises as to whether this system is the first to become activated when germination is begun The variations of the respiratory quotient with age for a number of seedlings with different food reserves, among them being *Lupinus luteus*, suggest that the respiratory quotient is to a certain extent an indication of the relative intensities of utilization of fats and carbohydrates (Stiles and Leach (1933)) Consequently it is desirable to have data for *L albus* as to oxygen consumption and carbon dioxide production at the beginning of germination The procedure followed was to make determinations with the Warburg microrespirometer on single seedlings chosen from groups brought to successive stages in germination under identical conditions It has been found that the respiratory quotient 1 hour after putting the seeds to soak in water was 1 00 The quotient then fell to 0 76 in 9 hours, rose to 0 90 at 12, and fell gradually to 0 64 at 60 hours An initial respiratory quotient of unity removes the fat oxidation from consideration as the first source of energy, but in view of the sharp drop during the first few hours, it is probable that it does come into play soon after germination has begun

II

The seeds used in these experiments came from a strain of *Lupinus albus* L inbred for 3 years by Dr A E Navez Following the method of Tang (1930-31) the seeds were germinated by soaking them in water for 12 hours removing the testas, and incubating them in moist boiled maple sawdust at 20° in the dark

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The 21, 36, 48, and 60 hour seedlings were treated in this manner, but the 6, 9, and 12 hour seedlings were soaked in water for those periods and after the removal of the testas, placed directly in the respirometer vessels. In the case of the 1 hour seedlings, the testas were removed before the seeds were put to soak

Oxygen consumption and carbon dioxide production were measured on six seeds in each age group with the apparatus described by Tang (1931–32). The net pressure change due to both oxygen consumption and carbon dioxide production, with no alkali in the vessel, was measured at 15 minute intervals for an hour, 0 2 ml of 10 per cent NaOH was then placed in the side arm, and the pressure change due to oxygen consumption alone measured at 15 minute intervals for an hour. The pressure change due to carbon dioxide production is the difference between the two 1-hour readings. The assumption underlying this

TABLE I

Successive 15 Minute Readings

	9 hr seedlings					
	1	2	3	4	5	6
$\dfrac{\Delta h\ O_2}{15\ min}$	11 10 12 8	12 10 12 8	12 10 13 9	13 9 13 9	12 8 12 8	13 9 13 9
	36 hr seedlings					
	1	2	3	4	5	6
$\dfrac{\Delta h\ O_2}{15\ min}$	21 22 20 22	20 20 18 20	26 25 25 26	26 26 25 27	25 25 24 25	19 19 17 20

method is that oxygen consumption continues at the same rate in both periods. This is justified to a certain extent by the small amount of the variation in the 15 minute readings. Data from the experiments on the 9 and 36 hour seedlings are given in Table I in terms of millimeters of Brodie solution. All measurements were made in the dark at 20° ± 0 05

III

The average rate of oxygen consumption after the seeds have soaked in water 1 hour with the testas removed was 29 mm [3] per hour per seed. From this point, the rate rose to an average of 246 at 60 hours. The rate of carbon dioxide production did not follow closely that of

oxygen consumption, so that there were fluctuations in the respiratory quotient For the 1 hour seedlings the respiratory quotient had a value of 1 0 with an average deviation of 6 per cent The value then
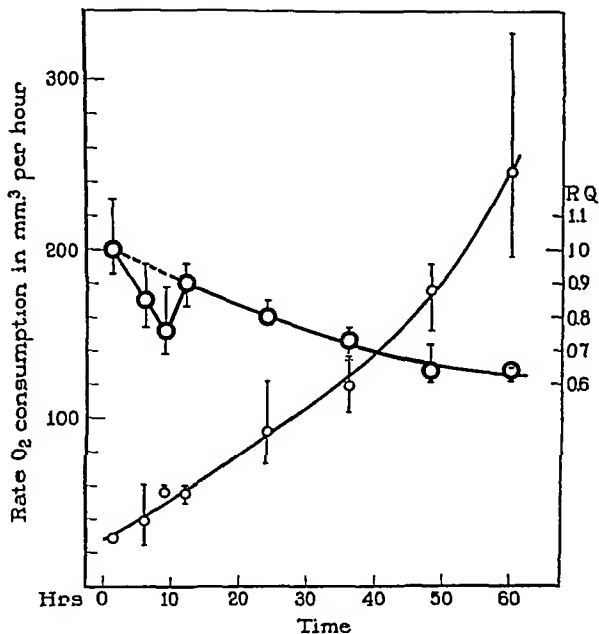
FIG 1 Large circles represent respiratory quotients, small circles, rates of oxygen consumption as mm³ per hour per seed The bars indicate the maximum deviations from the mean

fell to 0 76 for the 9 hour seedlings There was more variation in the 6 and 9 hour stages and the two values above unity in these two groups are not regarded as significant and do not appear in the means plotted in the figure After a rise to 0 90 at 12 hours, the respiratory quotient

fell again to 0 61 at 60 hours   The means and limits of variation for both oxygen consumption and the respiratory quotient are plotted as functions of time from the beginning of soaking, in Fig 1

<div align="center">IV</div>

As Stiles (1935) has remarked in connection with the respiration of the potato tuber, "   if only sugar or some other carbohydrate is utilized, and if this is oxidized completely to carbon dioxide and water, and if there is no internal source of oxygen, and if all the carbon dioxide produced escapes from the tissues, then the respiratory quotient should be unity "   The observation of an initial respiratory quotient of 1 0 does not prove that these conditions have been satisfied   Nevertheless, it is a good indication that the first substrate utilized in the germination of *Lupinus albus* seeds is a carbohydrate

A comparison of the data on *L luteus* (Stiles and Leach (1933)) and *L albus* shows that the variations of the respiratory quotient with age are of the same type   The earliest observed values are about unity, in the first 12 hours, the respiratory quotient falls and rises again to a slightly lower level, for the next 48 hours, it slowly declines   There is, however, a distinct difference between the two lupines, although the values of the respiratory quotient nearly coincide at the onset of germination and at the 12 hour stage, the quotient of *L albus* is lower than that of *L luteus* at other points   In the 9 and 48 hour stages, the respiratory quotients were 0 76 and 0 64 respectively in *L albus* and 0 9 and 0 76 respectively in *L luteus*   Such a result would be expected on the theory that the respiratory quotient depends in great part on the relative intensities of utilization of carbohydrates and fats, for according to the data of Guillaume (1923) the fat contents of seeds of *L albus* and *L luteus* are 8 88 per cent and 4 17 per cent respectively

In discussing the respiration of germinating seeds it should be remembered, as Kostychev (1927) points out, that simultaneously with oxygen respiration there is also an independent absorption of oxygen for other purposes   For example, McKie (1931) has shown that in the 8th day of germination of *L luteus* seeds, 20 per cent of the nitrogen present in the ungerminated seeds has been converted into asparagine, which is not found in the ungerminated seeds   According to Palladin (1889), this is formed only in the presence of oxygen, so that

utilization of fat may not account entirely for the low respiratory quotient in the later stages

SUMMARY

In germinating seedlings of *Lupinus albus,* the initial respiratory quotient was found to be unity   After a drop to 0 76 at 9 hours, the value rose to 0 90 at 12 hours, and then fell to 0 64 at 60 hours   It is improbable that the fat oxidation system is the first to become activated

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COAGULATION OF MYOSIN BY DEHYDRATION

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The coagulation of myosin that occurs when muscle contracts, or when it stiffens in rigor, differs from the coagulation of myosin and other proteins produced by denaturing agents, such as heat, acid, alkali, urea, ultraviolet light, and surface action (Mirsky, 1935–36) When proteins become insoluble due to the effect on them of any of these agents, there is an accompanying change in their sulfhydryl and disulfide groups (Mirsky and Anson, 1935–36)   Groups that are not detectable in the native state become detectable when the protein is denatured, that is, when after being treated with certain agents it becomes insoluble under conditions under which it was previously soluble   No change in its SH groups takes place, however, when myosin becomes insoluble in rigor   It has recently been found that in fertilization a similar change occurs in the proteins of the egg (Mirsky, 1936), as in muscle a protein becomes insoluble without a change in groups   In muscle and in the egg as much as a third of the total protein may coagulate   It is important for an understanding of the changes that take place in muscle and in the egg to find a type of coagulation that occurs *in vitro* in which loss of solubility is *not* accompanied by changes in SH and S S groups

In the coagulation of myosin caused by dehydration, it will be shown in this paper, loss of solubility occurs without a change in groups, and in this respect coagulation by dehydration is, of all the many ways of coagulating a protein *in vitro*, the only change in protein known to resemble that which has been observed to occur in the cell Water is removed from the protein by both freezing and drying, that is, by subjecting the protein to a low temperature in a vacuum desiccator which is continually evacuated   The kinetics of the coagulation process have not been studied

There are in the literature a few scattered references to the effect of

drying on the properties of protein   Some proteins lose their solubility by being dried, many do not   The reason for this difference is not clear   Hemoglobin and the serum proteins can be dried without becoming insoluble (Hardy and Gardiner, 1910) but thyroglobulin becomes insoluble if it is dried (Heidelberger and Pedersen, 1935–36) In some proteins, the yellow oxidizing ferment and the "Zwischenferment" of Warburg, if there is any change in configuration on drying, it must be very small, for after drying these proteins cannot only be re-dissolved, but it is found that their enzymatic activities are unimpaired (Theorell, 1935, Negelein and Gerischer, 1936)   On the other hand, if a casein preparation observed to be monodisperse in the ultracentrifuge is dried and then re-dissolved, it is found to be polydisperse (Carpenter)   The effect of dehydration on the x-ray diffraction photographs obtained from proteins has been investigated by Bernal and Crowfoot (1934), by Astbury and Lomax (1935), and by Astbury, Dickinson, and Bailey (1935)

Dehydrated myosin is dissolved less readily than is myosin which has been denatured by acid   Myosin denatured by acid readily dissolves in 0 01 N hydrochloric acid and, on the other side of the isoelectric point, in 1 2 M potassium chloride solution at pH 8   If dehydrated myosin is kept in these solutions in the cold for as long as 4 weeks no more than a trace dissolves   When myosin coagulates in muscle, it too is insoluble at pH 8 and in 0 01 N hydrochloric acid

The SH groups of the following soluble and insoluble preparations of myosin were investigated native soluble myosin, myosin denatured by acid, myosin coagulated by dehydration, myosin coagulated by dehydration, and then treated with acid   SH groups were estimated by their reaction with iodoacetate, using a method that has been previously described (Mirsky and Anson, 1934-35)   In this procedure myosin is treated with an excess of iodoacetate, subsequently hydrolyzed, and the cysteine content of the hydrolysate estimated The decrease in cysteine content as compared with that of protein not treated with iodoacetate is a measure of the number of "active" SH groups detectable in the protein before it was hydrolyzed   In a native protein the number of detectable groups depends upon the pH All estimations were made at pH 7 6 because in this region slight variations in acidity do not affect SH groups and because at pH 7 6

iodoacetate reacts with all the SH groups of myosin denatured by acid
The coagulum obtained by dehydrating a frozen myosin solution is of
such a delicate, filmy texture that it is unlikely that any serious barrier
is offered to iodoacetate as it penetrates the protein

TABLE I

*SH Groups, Expressed As Percentage of Cysteine, of the Various Myosin
Preparations*

Protein	Cysteine content before treating with iodoacetate	Cysteine content after treating with iodoacetate	Active SH groups. (1) minus (2)	Cysteine content after denaturing with trichloracetic acid and then treating with iodoacetate	Active SH groups after denaturing with trichloracetic acid (1) minus (4)
	1	2	3	4	5
Soluble myosin	0 47	0 24	0 23	Practically none	0 47
Myosin coagulated by freezing	0 47	0 23	0 24	Practically none	0 47
Myosin coagulated by drying	0 44	0 23	0 21	Practically none	0 44

EXPERIMENTAL

The myosin used in these experiments was prepared from cow muscle   The
method of preparation differed only slightly from that described by Howe (1924)
and Edsall (1930)   Instead of precipitating myosin by dilution with water, it was
found more convenient to precipitate it by placing the solution in a cellophane
tube and dialyzing against distilled water   The final precipitate was washed with
0 1 M NaCl to shrink its volume   Part of the precipitated myosin was placed in a
chamber at −25 for 3 days   Another part was placed in a desiccator at −3
where it was continually evacuated with a pump for 36 hours   A sample subse
quently dried in an oven at 110° lost 7 per cent of its weight   A swollen, salt free
precipitate of myosin was placed in the cold evacuated desiccator   This prepara
tion clung very tenaciously to its water, and only after a week did the myosin ap
pear to be dry   It was found to be insoluble   When the salt free myosin which
appeared to be dry was heated to 110° it lost 50 per cent of its weight within a few
minutes and no more on more prolonged heating

To test the solubility of both the myosin frozen at −25° and that dried at −3
samples were placed in 0 01 N HCl and in 1 2 M KCl brought to pH 8 0 by addi
tion of M K₂HPO₄   These suspensions were kept at 0 for 4 weeks   The super

natant fluids at the end of this time gave only a slight turbidity on addition of an excess of trichloracetic acid, indicating that only a trace of protein dissolved Soluble native myosin coagulated by trichloracetic acid quickly re-dissolved in both 0 01 HCl and in alkaline 1 2 M KCl

The method of estimating SH groups of myosin by their reaction with iodo-acetate has previously been described (Mirsky and Anson, 1934-35) All reactions with iodoacetate were in pH 7 6 phosphate buffer The results of the final meas-urement can be known qualitatively after the reaction with iodoacetate by testing the various protein samples with nitroprusside and ammonia Preparations that were treated with acid and then with iodoacetate gave no color with nitroprusside, dehydrated myosin gave a fairly intense color, although not as intense as before the reaction with iodoacetate

RESULTS

The conclusion to be drawn from these results is that, despite a certain resemblance, coagulation by dehydration is a distinctly different phenomenon from the coagulation produced by such agents as heat and acid Wherein the difference lies has recently been indicated in a general theory of denaturation and coagulation (Mirsky and Pauling, 1936) In denaturation the configuration of the molecule is profoundly changed, and as a consequence the protein becomes less soluble, readily forming a coagulum In dehydration, when the shell of water surrounding the protein particle is removed, the outer groups of the particle unite with the outer groups of other particles On restoring water these bonds may be readily broken so that the protein re-dissolves, as in methemoglobin and the serum proteins, and in some proteins (such as the yellow ferment) if there is any disturbance at all in the configuration of the molecule, it is trivial In myosin the bonds formed between protein particles by dehydration remain even when water is restored, and, although there is probably some change in internal configuration, the behavior of SH groups shows that the change is far less pronounced than after the action of heat or acid If native myosin, like other native proteins, is considered to have a special configuration, it would appear that after dehydration myosin still has a special configuration, possibly somewhat different, however, from that of native myosin, whereas after treatment with heat or acid only the "debris of the original special configuration" remains

The importance of coagulation by dehydration for an understanding of coagulation in the egg and in muscle is that by an exceedingly simple

procedure there is produced a change in the isolated protein which resembles the change that occurs in protein while it is still part of the living cell   The significance of this resemblance will be discussed in a paper on the coagulation of myosin in muscle

SUMMARY

When myosin is dehydrated it becomes insoluble   The number of detectable SH groups in myosin coagulated by dehydration is the same as in native soluble myosin   In this respect coagulation by dehydration differs from coagulation brought about in any of the other ways now known, but resembles the coagulation that occurs in muscle during rigor and in the egg after fertilization

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THE COAGULATION OF MYOSIN IN MUSCLE

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The coagulation of myosin is one of the few changes in the proteins of muscle known to take place during contraction and rigor   Although under certain conditions as much as one third of the total protein of muscle may become insoluble, the significance of this change for the shortening of muscle is not understood   And yet the recognition of a definite transformation in the substance of muscle should be of value in investigating the mechanism of contraction, especially when one recalls that nearly all of those abortive theories of contraction that have been formulated since the time of Descartes have been based on knowledge of systems supposed to be analogous to muscle, rather than on a knowledge of the properties of living muscle itself   As a step towards an understanding of the chemical properties of the "living machinery" of muscle (as distinguished from the metabolic trans formations in muscle) I have, accordingly, investigated the coagulation of myosin   I have already shown how the coagulation of myosin in muscle is related to the denaturation and coagulation of isolated myosin (Mirsky, 1935–36 and 1936–37)   By measurements of protein sulfhydryl groups it was found that the coagulation of myosin in muscle differs from the coagulation of myosin and other proteins brought about by the usual denaturing agents (such as heat and acid) but resembles the coagulation of myosin caused by dehydration   At this point it is important to recall that when myosin is said to coagulate in muscle it is not supposed that myosin actually precipi tates from solution   In muscle probably only a very small part of the myosin present is dissolved (Smith, 1934)   That there is a change in myosin in muscle is inferred from the fact that at one time the pro tein can be dissolved in certain media in which at another time it

cannot be dissolved, the myosin is said to have coagulated   Apparently myosin in muscle can pass from one gel state to another   How these states differ will be considered in this paper

Powdered Muscle —The first need is to find a method by which coagulation can be investigated under conditions more subject to control than those obtaining in intact muscle   It was formerly supposed that a rapid and spontaneous coagulation of myosin occurs in the juice expressed from minced muscle (Kuhne, 1864)   Recently, however, Smith (1930) showed that the myosin precipitated under these conditions re-dissolves on adding salt and maintaining the solution faintly alkaline   When, however, myosin coagulates in muscle it cannot be dissolved in a faintly alkaline salt solution   Since coagulation resembling that which occurs in muscle does not spontaneously occur in juice expressed from muscle, I decided to begin with a preparation much closer to intact muscle   Muscle in the form of a dry powder was made in the following way, the hind legs of a frog were cooled to 0°, the muscles were then removed, finely minced, placed in a vacuum desiccator at about $-8°$ and dried *in vacuo* while frozen, the dried material was ground to a powder in a mortar   This dry powder has some of the properties of live muscle   The myosin in it is still soluble, as in intact muscle, and, more important still, under certain conditions the myosin can be caused to suddenly coagulate   Furthermore, the myosin in this preparation is stable, it can be kept for several months, at least, without undergoing more than slight changes   It is therefore convenient to prepare large quantities of the material, a uniform powder being obtained from many different frog legs

If a quantity of water equal to that previously removed is added to the dry muscle powder, the myosin in it instantly coagulates   Deuticke (1930) has observed that myosin loses its solubility after a normal contraction as well as after rigor   The speed with which coagulation occurs in my preparation indicates that probably a similar change takes place in it as in the contraction of intact muscle   Further evidence that the change in myosin which takes place on wetting the powder is substantially the same as that which occurs in contraction is provided by an observation made by Hurthle twenty-seven years ago (1909) in his classical paper on the minute anatomy of muscle

Hurthle observed that in dried insect muscle the anisotropic layer is of the thickness characteristic of resting muscle, but that on wetting, this layer diminishes in thickness as it does during contraction The properties of powdered muscle which I have described are sufficient to

TABLE I

Percentage of Soluble Protein in Various Preparations of Powdered Muscle

	Residual protein	Extracted protein	Percentage extracted
	gm	*gm*	
Preparation 3 dry powder extracted with KCl	0 30	0 67	69 0
Preparation 4 dry powder extracted with KCl	0 25	0 655	72 4
Preparation 6 dry powder extracted with KCl	0 252	0 597	70 3
Preparation 3 5 cc water added and the mixture allowed to stand at 20 for 30 min Extract with KCl	0 47	0 37	44
Preparation 3 5 cc water added and the mixture allowed to stand at 20 for 30 min Extract with 0 01 N HCl	0 45	0 402	47 3

TABLE II

Fractionation of Protein Extracted from Preparation 3 before and after Coagulation, Using Approximately 1 Gm of Powder for Each Experiment

	Residual prot in	Myosin	Myogen	Total extracted protein	Total protein	Protein extracted	Myosin	Myogen
	gm.	*gm*	*gm*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
Before coagulation	0 237	0 305	0 163	0 468	0 705	66 4	43 2	23 1
After coagulation Add 5 cc. water and stand at 20 for 30 min	0 482	0 096	0 203	0 299	0 781	38 3	12 3	26 0

show the usefulness of this preparation, its limitations will appear as it is used

Wetting the powder causes an immediate change in myosin, but this change does not occur in the presence of much salt The system remains stable, therefore, when wetted by a concentrated salt solution,

and on this depends the method of estimating the relative quantities of soluble and coagulated myosin   The procedure for estimating the quantity of soluble protein is to add a large volume of cold 1 2 M KCl, make the suspension slightly alkaline with a little K_2HPO_4 and stir in the cold for several hours   More protein is not extracted by more prolonged stirring   After centrifuging, the quantity of the protein in the supernatant is estimated   The soluble protein can also, of course, be fractionated and the several constituents estimated separately   In one experiment the quantity of soluble protein after coagulation was estimated by extraction with 0 01 N HCl,[1] the result being about the same as after extraction with KCl

The fact that muscle can be dried without thereby coagulating the myosin in it is surprising if one recalls that isolated myosin is coagulated merely by drying (Mirsky, 1936–37)   Apparently myosin in muscle differs in some way from isolated myosin, and the difference is important for an understanding of the coagulation of myosin in intact muscle   Fibres of dried isolated myosin have been made by Weber (1934) and after allowing them to swell in a physiological salt solution he considers them to be an exact model of the fine structure of the muscle fibre, or rather of its anisotropic band   The similarities between a muscle fibre and a myosin fibre observed by Weber are certainly noteworthy, but the difference discovered when dried, *live muscle* is compared with dried myosin is significant for this difference is not observed if *muscle* in *rigor* is dried and compared with dried myosin   The difference (which is detected when muscle is dried) between the states of myosin in living muscle and in a fibre made of isolated myosin is important because it is associated with the irritability of muscle

Nature of Myosin Coagulation —Various characteristics of the

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[1] Dilute HCl was used because, according to Smith, it extracts more protein from minced resting muscle than does salt   Smith states that he has been unable to detect any coagulation of myosin as a consequence of rigor mortis   His results, which I have seen in abstract only (*Chem Abstr* , 1936, **30**, 496), contradict the results reported in this paper and elsewhere   He attributes the contradiction either to the strict maintainance of a low temperature during storage of the muscle or the absence of antiseptics in his experiments   In most of my experiments, however, the muscle was kept at 0° or below, and no antiseptics were used

coagulation of myosin have been investigated, using preparations of powdered muscle

1 The Kinetics of Coagulation—This was studied by restoring to the muscle powder the water removed in drying and estimating at various intervals of time thereafter the quantities of soluble protein present   The speed of the process is striking, at 20° more than one half of the myosin in muscle coagulates within a minute   At lower temperatures the change occurs more slowly   The temperature coefficient, approximately 2, is more characteristic of a chemical than

TABLE III

Coagulation Produced by Adding to 1 Gm of Powdered Muscle 5 Cc of Water, Allowing the Mixture to Stand for Various Lengths of Time and Subsequently Extracting with 1 2 M KCl

Time	Percentage of soluble protein			
	0	10°	20°	0° adding 5 cc. of 0 02M Na₂CrO₄ instead of water
Dry powder extracted immediately	70 0			
1 min	70 0		52 8	
2½ min	66 0	54 5	49 8	
5 min	61 6	53 6	45 4	59 8
15 min	56 1	51 0		
30 min	54 3			54 9
60 min	52 6			
120 min	51 2		44 0	48 9
240 min	47 4			
22 hrs	44 8			

of a physical process   But when water is added to dry muscle many factors are involved, and it is unlikely that the resulting change can be described in terms of a single chemical reaction

Another way of bringing about coagulation is to add the powder to a large volume of dilute saline   1 gm of powder may be stirred into 500 cc of solution   If no salt is present coagulation does not occur under these conditions (the myosin remains in the muscle but it can be subsequently extracted) and if too much salt is present, myosin does not coagulate because it is extracted   Within these two limits there

is room for considerable variations in salt concentration   In 0 006 м NaCl not much coagulation occurs, in 0 02 м NaCl more occurs, and in 0 1 м about the same amount occurs   Other salts can be used In a 0 02 м phosphate buffer practically all the myosin coagulates Varying the pH from 6 15 to 7 35 made no apparent difference in the amount of coagulation   At 0° under these conditions the change is

TABLE IV

Myosin Coagulated by Adding 1 Gm of Powdered Muscle to 500 Cc of Dilute Salt Solution and Subsequently Extracting with 1 2 м KCl

	Time	First extraction	Extracted subsequently with 1 2 м KCl	Residual protein	Percentage of total protein extracted	Percentage of protein extracted by 1 2 м KCl (first protein extracted not reckoned in total)
	min		*gm*	*gm*		
Water	60	0 247	0 282	0 296	64 2	48 8
0 006 м NaCl	5	0 133	0 325	0 341	57 3	48 8
0 02 м NaCl	5	0 135	0 263	0 414	49 0	38 9
0 02 м KCl	5		0 344	0 345	49 9	
0 1 м NaCl	5		0 322	0 305	51 4	
0 1 м KCl	5		0 343	0 339	50 4	
0 02 м KHPO₄ buffer pH 6 15	5	0 119	0 232	0 423	45 4	35 4
pH 6 15	30	0 142	0 238	0 438	46 5	35 4
pH 6 55	5	0 121	0 216	0 420	44 7	34 0
pH 6 55	30	0 160	0 190	0 430	44 9	30 7
pH 7 05	15	0 172	0 206	0 451	45 6	31 4
pH 7 35	5	0 141	0 207	0 431	44 7	32 4
First add 20 cc of 1 5 м KCl and stand for 40 min Then add 480 cc H₂O Final concentration 0 06 м KCl	5	0 215	0 302	0 234	68 9	56 5

practically complete in less than 5 minutes   This is much more rapid than when the powder at 0° is moistened with a quantity of water equal to that removed in drying

2 The Effect of Oxalate —This was investigated because the coagulation of myosin suggests comparison with the clotting of fibrinogen In the formation of fibrin the rôle of calcium is concerned with the activation of thrombin   It has frequently been supposed that calcium

plays a similar rôle in the coagulation of myosin Experiment shows, however, that myosin readily coagulates in the absence of free calcium When a dilute oxalate solution is added to powdered muscle, coagulation occurs just as it does when water is added (see Table III)

3 Removal of Water-Soluble Constituents —It is possible to thoroughly extract powdered muscle with water without thereby coagulating more than a fraction of the myosin Although restoring to the powder the quantity of water removed in drying results in rapid coagulation of myosin, if the powder is stirred into a large volume of cold water, most of the myosin remains within the muscle undissolved but still soluble When the residue, after extraction with water, is extracted with 1 2 M KCl more than half of the myosin dissolves Extraction with water can be prolonged, lasting for several hours, and the extracted material can be kept for at least 8 hours without any further change taking place in the myosin than occurred when the powder was first stirred into water Quantitatively the most important constituent of muscle extracted by water is the protein myogen, and all the myogen is extracted (along with a small amount of myosin) Myogen extracted in this manner constitutes 24 per cent of the total protein in muscle If, using 1 2 M KCl, myogen is extracted along with myosin from powdered muscle, and if the two proteins are then separated by dialysis, the same quantity of myogen is obtained as by simple extraction with water (see Table II) If, after extraction with water, the muscle is suspended in a dilute phosphate solution for a moment, quickly centrifuged, and then extracted with 1 2 M KCl, myosin is found to be coagulated

4 The Significance of Structure for Coagulation —Myosin must be in its proper place in the structure of the muscle fibre if the rapid coagulation described above is to occur, as the following experiment shows If to some muscle powder a small volume of relatively concentrated salt solution is added (to 1 gm of powder 20 cc of 1 5 M KCl) no coagulation occurs, for the salt is concentrated enough to dissolve myosin The mixture can be kept at 0° for at least an hour without any change in the quantity of soluble myosin The salt is now diluted by addition of a large volume of cold water (480 cc so that the final concentration of KCl is 0 06 M) If muscle powder is added directly to this volume of such a dilute salt solution coagulation occurs

immediately   In the diluted suspension of muscle, however, myosin does not coagulate, nor is it dissolved in the dilute saline, it is simply precipitated and can be dissolved by adding salt   Once myosin has been removed from its place in the muscle fibre, it does not coagulate as it previously did even if it is mixed with the rest of the muscle from which it was displaced (see Table IV)

5 *The Rôle of Water in Coagulation* —When muscle powder is treated with a volume of water, so large that no coagulation occurs,

TABLE V

1 gm of powdered muscle extracted with 500 cc of water and subsequently with 1 2 M KCl   In one experiment the water-extracted material was coagulated by freezing and in another experiment it was coagulated by mixing with a dilute phosphate buffer

	Protein extracted with water	Protein extracted with KCl	Residual protein	Total protein extracted	Protein extracted with KCl (not reckoning water extracted protein)
	gm	*gm*	*gm*	*per cent*	*per cent*
Experiment 1	0 247	0 282	0 296	64 2	48 8
Experiment 2	0 235	0 320	0 257	68 4	55 4
Experiment 3	0 240	0 288	0 330	61 7	46 6
Experiment 4	0 203	0 225	0 259	62 2	46 5
Freezing at −8° after extraction with water		0 340	0 295	53 6	
Mixing the water-extracted material with 0 02 M pH 6 55 phosphate buffer for 5 min at 0°	0 203	0 174	0 312	54 8	35 8

the muscle swells to a considerable volume   When a little salt solution is added coagulation occurs and there is at the same time a striking shrinkage in volume   1 gm of muscle powder swells to about 25 cc and then shrinks to about 7 cc   The correlation between loss of water and coagulation of myosin may be explained by supposing that as long as the myosin particle is enveloped by a shell of water as in swollen muscle it can be dissolved, but when that shell is removed the groups of one particle unite with those of neighboring particles to form an insoluble coagulum   It has already been shown that simply dehydrating isolated myosin by drying or freezing causes it to become

insoluble On the other hand, myosin in intact muscle is not coagu lated by freezing and drying Experiments on swollen muscle show that when this is frozen at $-8°$, the myosin in it is coagulated (see Table V) In this respect, then, myosin in swollen muscle behaves like isolated myosin and unlike the myosin of intact muscle But myosin in swollen muscle can be coagulated simply by adding a little neutral salt, and this extreme instability recalls the myosin of intact muscle rather than isolated myosin

DISCUSSION

On the basis of the new information presented in this paper and of other knowledge of myosin in muscle, it is perhaps possible to conceive of how the particles of myosin are arranged in muscle and what change occurs during coagulation

The coagulation of myosin in muscle bears a certain resemblance to coagulation of myosin caused by dehydration In neither is there that change in sulfhydryl groups characteristic of coagulation due to the familiar denaturing agents, such as heat, acid, urea, etc In neither is the coagulum soluble in dilute HCl, in which, however, myosin denatured by acid readily dissolves And in swollen muscle prepared by extracting powdered muscle with a large amount of water, in which myosin appears to be still placed somewhat as it is in intact muscle, myosin coagulates when dehydrated When myosin coagu lates due to dehydration the profound change in internal configuration caused by the usual denaturing agents does not occur The groups of one myosin particle combine with those of its neighbors to form a coagulum without causing more than a relatively slight alteration in internal configuration

The way in which myosin is imbedded in the structure of muscle is important for coagulation If myosin is dislodged from this position (as it is by addition of concentrated salt solution), that rapid coagula tion characteristic of muscle no longer occurs The significance of structure is also indicated by the fact that myosin in intact muscle does not coagulate on dehydration

A fairly satisfactory picture of the arrangement of myosin particles in muscle can be formed One of the layers of cross striated muscle has long been known to be doubly refractive On the basis of Wiener's

theory and the conceptions of Ambronn and Frey (1926), Stubel (1923) has shown that the anisotropic layer contains a multitude of minute rod-shaped particles (small compared with the wave-length of light) oriented with their long axes parallel to the axis of the muscle fibre   The anisotropic properties of myosin discovered by von Muralt and Edsall (1930) and by Weber (1934), make it probable that rod-shaped particles in muscle consist, in part at least, of myosin   The linear arrangement of myosin particles in muscle is also indicated by the observations of Rubner (1922) and Hurthle (1931) on the changes in dimensions of muscle on drying   When a muscle, such as the frog's sartorius, is dried there is a pronounced shrinkage in volume but its length diminishes only slightly, the great decrease occurring transversely to the axis of the fibres   The great mass of water in muscle appears to be located between longitudinally arranged strips of protein

If this picture of the fine structure of the anisotropic layer is accepted and if coagulation in muscle resembles the coagulation of myosin caused by dehydration, then it may be supposed that when myosin coagulates in muscle, the myosin particles join to each other end to end   In resting muscle the rod-shaped myosin particles are loosely arranged in a line end to end, and when coagulation occurs they string together to form a thread of myosin   This can be accomplished with speed because although there is much water separating the particles laterally, the ends of the particles are close to each other   If the particles are dislodged from their longitudinal arrangement, they are enveloped on all sides by water so that rapid coagulation can no longer occur

It is now necessary to consider why the myosin in intact muscle can be dried without coagulating   Possibly this myosin has a different configuration from myosin prepared by the present methods, however carefully they are carried out   Gorter (1936) has recently shown how the properties of myosin are markedly changed by a slight enzymatic hydrolysis   Some such change, it may be imagined, takes place during the extraction of myosin, and even in the moment when powdered myosin is merely moistened with water, and this modified myosin then coagulates   Another explanation would postulate a change not in the configuration of myosin but in the substance lying

between the ends of myosin particles   In intact muscle and in frozen and dried muscle the substance in this region prevents the ends of the particles from joining, but when powdered muscle is moistened this substance is changed, so that it no longer acts as a barrier between the ends of myosin particles   This substance must be in the form of a thin film, and the freezing of muscle, preceding drying, would probably change its structure so that subsequent wetting might destroy it Whatever the explanation of the stability of soluble myosin in muscle may be, it seems likely that a similar change occurs both in intact muscle during contraction and in muscle powder on wetting, a change which causes myosin to coagulate

EXPERIMENTAL

1 Preparation of Muscle in the Form of a Dry Powder —Muscles of the hind legs of frogs *Rana catesbiana*, were used   The hind legs were severed by one cut with a heavy cleaver just above the pelvis, and kept at 0° for 5 hours   The muscles were then rapidly dissected and finely minced in a cold room at 0°   The minced muscle spread in a thin layer on a large watch glass was frozen at −8°, and the rest of the procedure was carried out at this temperature   The frozen muscle was placed in a vacuum desiccator, containing anhydrous calcium chloride which was evacuated by a pump which ran continuously   After 24 hours the muscle was in the form of a fairly dry solid cake   This was removed from the watch glass broken up and then replaced in the desiccator   At the end of another 24 hour period the muscle was as dry as it can be made by this procedure   It was ground to a fine powder in a mortar and stored at −8°   This powder lost 3 6 per cent of its weight when dried in an oven at 110

The effect of the various steps in the procedure after mincing on the quantity of extractable protein was determined   The myosin in minced muscle is quite stable the quantity of protein extracted from muscle immediately after mincing is the same as after the minced muscle has stood at 0 for 2 hours   Freezing of minced muscle causes a slight change a few more per cent protein were extracted after freezing probably because the frozen tissue was more completely disintegrated Drying had no further effect   In eleven different preparations, all however made at the same time of the year —in March April and May the quantities of soluble protein varied from 69 0 to 72 8 per cent of the total protein   In the course of 3 months (from May 25, to September 4) the quantity of soluble protein in one preparation dropped from 70 0 to 68 per cent   How the other properties of pow dered muscle change with time is not known, since most of the experiments de scribed in this paper were performed on a large single preparation over a period of 3 months and there was no opportunity at that time of making a fresh preparation It is possible, therefore, that some of the properties of powdered muscle described

in this paper are due to the fact that the powder had been stored for some time, even though storage was at a low temperature

It was at first thought that a preparation more like that of intact resting muscle would be made if the tissue were frozen before mincing and then minced while frozen   Muscles were accordingly frozen at −8° and then finely minced without thawing   The minced frozen tissue was added to the extraction fluid which was rapidly stirred so that only a short time intervened between thawing of a particle and penetration of it by concentrated saline   Only 59 to 60 per cent of the total protein dissolved   It is well known that freezing a muscle serves as a stimulus to contraction, apparently the effect of freezing is much greater on intact than on minced muscle

Extraction —1 gm of powder was extracted at 0° with 240 cc of 1 2 M KCl and 4 cc of 1 M K_2HPO_4 in a 250 cc centrifuge flask   This solvent was used because Howe found that of the various salts he tried, potassium salts of this concentration were the most effective extractants   Extraction was aided by continual stirring and lasted 2 hours   No more protein was extracted in 6 hours   The pH of the mixture was about 8 0, just red to cresol red   After extraction under these conditions, stirring the residue for an hour in a 0 25 M pH 9 4 borate buffer failed to extract any more protein   The extracted and residual material were separated by centrifuging at high speed, a residue of less than 10 cc and a perfectly clear supernatant being obtained

Estimation of Protein —The extracted protein was precipitated by adding 20 cc of a 50 per cent trichloracetic acid solution (trichloracetic acid dissolved in an equal weight of water)   The suspension was centrifuged and the protein precipitate was freed of salt by washing twice, each time with 250 cc of a 5 per cent trichloracetic acid solution   The protein was dehydrated by stirring with 250 cc acetone and the suspension was centrifuged after adding to it 1 cc of concentrated HCl   Most of the acetone adhering to the precipitate was removed by suction, and lipoids remaining with the protein were extracted with 250 cc of a mixture containing two parts of alcohol and one of ether   The precipitate was transferred to a tared crystallizing dish and dried to constant weight at 110°   Since it was found that the relative quantities of extracted and residual protein were the same if the protein were dried after washing with acetone, in most experiments the treatment with alcohol-ether was omitted   The proteins in the residue after extraction were washed in the same way and then dried and weighed

In several experiments extracted protein was fractionated by dialysis   The supernatant fluid, after centrifuging, was poured into a long cellophane tube, 1 inch in diameter, which was placed in a rocking dialyzer   Inside the tube was a marble ball which rolled from one end to the other and thus stirred the contents   The extract was dialyzed in the cold against a continually flowing 0 01 M pH 6 6 phosphate buffer, for 16 hours   The contents of the cellophane tube, now partly precipitated, were centrifuged   The precipitate, which readily dissolved in 1 2 M KCl at pH 8 was considered to be myosin   The supernatant, which remained clear on more prolonged dialysis, was considered to be myogen   A search was made for globulin X, a protein in mammalian muscle described by Weber as being

insoluble in absence of salt but requiring for solution much less salt than does myosin   None however was found

Coagulation —To 1 gm of powdered muscle was added 5 cc of water   The powder was readily wetted by the water, and by mixing with a glass rod, the muscle was in a few moments of the consistency of a dough   In experiments at definite temperatures both powder and muscle were cooled, the powder being kept in a stoppered tube to prevent condensation of moisture on it   At any given time coagulation was interrupted by adding 235 cc of cold 1 2 M KCl

In experiments on the effect of oxalate, to 1 gm of powder was added 5 cc of 0 02 M sodium oxalate at 0   The calcium content of frog muscle is less than 0 0025 M

When muscle was stirred into 500 cc of a dilute salt solution, the solution was at 0°   The mixture was stirred for 2 minutes and then centrifuged the total time taken from adding the powder to pouring off the supernatant fluid being 5 minutes   The residue was extracted with 1 2 M KCl in the usual manner   The protein in each of the two extracts (in dilute saline and 1 2 M KCl) was precipitated and estimated separately

Extraction with Water —1 gm of powder was stirred into 500 cc of water at 0°, and the stirring was continued for an hour   After centrifuging, the residue was observed to be greatly swollen, volume 25 to 35 cc   By stirring with another 500 cc of water, no more protein was extracted   In some experiments the residue was extracted with 1 2 M KCl, in another it was frozen at −8° for 12 hours, and then extracted with 1 2 M KCl   And in others it was suspended in 225 cc of a 0 02 M pH 6 55 phosphate buffer, stirred for 5 minutes so that the myosin present coagulated centrifuged and the residue extracted with 1 2 M KCl all the extracted protein being precipitated and estimated

An attempt was made to measure the speed with which coagulation occurs when dilute phosphate is added by suspending the washed muscle in 225 cc of water adding a little phosphate and then immediately thereafter sufficient concentrated KCl to make the final concentration of KCl 1 2 M and so extract soluble protein It was found that even without adding dilute phosphate, addition of concentrated KCl to the suspended muscle caused coagulation   Coagulation also occurred if the aqueous suspension of washed muscle was poured into concentrated KCl solution   In the experiments described above for extraction 225 cc of 1 2 M KCl is added to water-extracted muscle compressed to 25 cc by centrifuging, and under these conditions much less coagulation occurs   This curious situation requires further investigation

The quantities of myosin and myogen extracted from muscle with water were estimated after dialysis as described above

SUMMARY

1 Muscle can be prepared in the form of a dry powder in which myosin exists in a state similar to that in intact muscle   As in intact

muscle, myosin in powdered muscle is soluble and can be caused to rapidly coagulate

2 Restoring to powdered muscle the quantity of water previously removed causes coagulation of myosin   The rate of coagulation is considerably slower at 0° than at 20°

3 Adding the powder to a large volume of dilute salt solution also results in coagulation

4 The water soluble constituents of muscle can be removed from the powder without thereby causing coagulation   Coagulation occurs in water extracted muscle when it is suspended in a dilute salt solution

5 Coagulation of myosin in muscle resembles the coagulation of myosin caused by dehydration

6 Myosin coagulates readily only when it is imbedded in the structure of muscle   The significance for coagulation of the arrangement of myosin particles in muscle has been indicated

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A PHASE RULE STUDY OF THE PROTEINS OF THE BLOOD SERUM A COMPARISON OF THE PROTEINS OF HUMAN, RAT, AND HORSE SERUM*

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In the course of the present investigation an effort has been made to ascertain whether the protein fractions of blood serum may be considered individual proteins They have been studied in various mammals and in both sexes

Previous attempts to separate individual proteins from blood serum by classical methods, even when taking the precaution to handle them quickly and at a low temperature, had led to the conclusion that the fractions, with the exception of crystalline serum albumin and possibly one globulin, were either not single proteins or were too unstable to behave as individuals In order to investigate individual unchanged proteins it seemed necessary to study the whole of the salting-out curve of *fresh* serum at 0° and to interpret it in the light of the phase rule

The fixing of pH and temperature are necessary prerequisites for the analysis of this question of the individuality of the proteins, since only after eliminating a sufficient number of variables can the phase rule be applied in answering this question [1]

For the application of the phase rule in its usual form to protein solutions certain assumptions are necessary Let us consider the mathematical statement C plus 2 $= P$ plus F where $C =$ the number of components, $P =$ the number of phases, and $F =$ the number of degrees of freedom or variance If the pressure is left at barometric pressure throughout, we have as variables only the composition of the

[1] See the work on egg albumin of Sørenson, S P L , *Compt rend trav Lab Carlsberg*, No 12

liquid phase and the temperature   There are as possible variables or degrees of freedom one less than the number of components in the liquid phase plus the temperature   In case the pH is left constant, the components are salt, H_2O, and protein   We have then three components and, if we assume one solid protein phase, three phases which give us then, two degrees of variance or freedom   If the protein is a single component and we fix two variables, such as the temperature and the concentration of the salt in the liquid phase, then according to the phase rule the concentration of the protein will be fixed regardless of the amount of protein in contact with the solution

If, however, more than a single protein is present, fixing the salt content will not fix the composition of liquid until there are as many solid protein phases in contact with the liquid as there are protein components

Thus, each new solid phase appearing when the salt concentration is increased will give a break in the continuity of the curve representing the liquid   Several such breaks appear on the diagram

These solid phases must be individual proteins, solid solutions of one or more proteins in another, or continuous series of compounds

Method

All experiments were carried out at 0° and at a constant pH (6 8 except where otherwise noted) which was checked by the glass electrode in the equilibrium liquid

Many previous qualitative experiments had raised misgivings as to the advisability of using $(NH_4)_2SO_4$ because of evidence of denaturation as indicated by the gradual clouding of the supernatant liquid and a progressive insolubility of the protein when the solutions are repeatedly brought from 0° to room temperature   Of other salts, potassium citrate, was most satisfactory in that a solution saturated with respect to protein at 0°, remained clear for at least a week, and any protein which separated upon repeated warming to room temperature dissolved when the solution was cooled to 0°   Previous experience in the extraction of pectin from fruit had impressed one of the authors with the specific effect of ions in causing an alteration in a colloidal substance   Such acids as citric or sulfurous could be used to extract an unchanged pectin, while hydrochloric or sulfuric acids at the same pH brought about a loss of its original properties. Potassium citrate is an excellent buffer   It is also sufficiently soluble at 0° to provide nearly complete salting out of the protein

The pH was brought to 6 8 by the addition of N/2 solution of citric acid This pH is well within the stability range as found by Svedberg and not too far from the pH of blood Then, the serum was dialyzed against a cold 5 per cent potassium citrate solution of pH 6 8, and was finally brought to the same pH by further addition of citric acid if necessary Rapid dialysis was carried out as suggested by Simms and Northrop in rocking Visking cellulose membranes in which a marble was included to stir the serum, until the dialyzing liquid gave no test for chloride and then for at least an half hour more Usually about 18 liters of dialysate were used for 50 cc of serum

All measurements were by weight The protein was determined by the gravimetric method of Barnett, Jones, and Cohn[2] with the modification that the salt was removed from the heat coagulated samples by washing well with the hot buffer solutions before adding ether and alcohol washes

Potassium was determined as potassium sulfate by adding an excess of sulfuric acid to the sample of serum, heating to dryness slowly on a hot plate to avoid spattering igniting at a low red heat in a muffle furnace to remove organic matter, and weighing

Known quantities of potassium citrate (Kahlbaum s) were added to weighed portions of serum in sufficient amounts to bring the total composition into the range of precipitation From these values the per cents of protein and potassium citrate in the mixtures were calculated Both the citrate and the citric acid added to maintain the same pH were finely ground to avoid mechanical occlusion of coated undissolved particles in the precipitate, and care was taken that the mixtures were very thoroughly stirred The tubes were agitated 3 hours at 0 in an ice water mixture to assure complete precipitation Previous experiments with $(NH_4)_2SO_4$ had shown that the equilibrium satisfied the most stringent test, it is the same whether it is attained by adding salt to the protein solution or by salting out the whole of the protein in a solution, separating and discarding the mother liquor and adding water and salt to the precipitated protein provided in both cases the final total composition is the same

Because of the instability of the protein solutions all processes were carried out as speedily as possible The solutions were filtered on funnels which were tightly covered with watch glasses or ground glass plates Weighed samples of filtrate were analyzed for potassium and protein The precipitated solid phases which separated at each point were rapidly pressed between filter paper to remove excess liquid, mixed as thoroughly and quickly as possible with a spatula, and preserved in glass stoppered bottles Samples were weighed dissolved in water and analyzed for protein and potassium

The results on the solid phases were necessarily inaccurate because of the difficulty of avoiding evaporation and of mixing the samples properly However, the fact that the plotted points of solid phase,

[2] Barnett, C W, Jones, R B, and Cohn R. B, *J Exp Med*, 1932, 55 633

total composition, and liquid phase, so nearly fulfill the theoretical requirement that they fall on a straight line when plotted according to per cent composition makes it impossible to disregard their significance   The probable error of the variation from the mean of two determinations of protein as found from about three hundred duplicate analyses of protein solutions is 0 007 parts per part of protein present

The complete reproducibility of the results is shown in Fig 3 where the data from two samples of serum, from different groups of 40 rats under the same dietary conditions, are plotted in the same diagram

All data are recorded upon the Gibb's triangle[3] in terms of per cent

[3] The Gibb's equilateral triangle was employed in which the three corners represent the three pure components   Any point within the triangle denotes a substance or mixture containing all three components

The perpendicular from each corner (100 per cent of the component) to the side opposite representing substances or mixtures containing none of that component may be used as a scale for measuring the per cent of the said component The geometrical theorem which states that the sum of all perpendiculars from any point in an equilateral triangle to its sides is equal to the altitude of the triangle, offers the means of measuring the per cents of all three components the sum is then equal to 100 per cent   For instance 100 per cent protein is found at the apex of the triangle   A point representing 10 per cent protein would fall on a line parallel to the base and cutting the altitude and the two sides 1/10th of their lengths from the base   Similarly, if the analysis showed 10 per cent salt, the point would have to be on a line 10 per cent of the distance from the left hand edge of the triangle to the right corner, measured either on the perpendicular to the side opposite or on one of the two sides which it cuts   In practice the protein in per cent by weight is measured along the left edge and the salt along the base The graph may then be viewed and the per cents of protein and salt read off just as if rectangular coordinates were used   The per cent of H_2O may be similarly located on a line parallel to the right side of the triangle

The X's on each diagram represent the per cents by weight of the components in the composition of each mixture of serum, dry potassium citrate, and citric acid Each point is calculated from an analysis of the serum and the added salt and acid   The boldly drawn curves in the lower parts of the diagrams represent the solubilities of protein at definite salt concentrations, since the curve separates the homogeneous liquid between it and the lower boundary of the diagram from the heterogeneous mixture of liquid and solid above   The distance, then from the X representing the total composition of a mixture and the corresponding O representing the analysis of the liquid separating from it is a measure of the protein precipitated at that point   The triangles represent the analyses of the

potassium citrate, per cent total protein, dried at 110°, and per cent H_2O (including any added citric acid)

In some cases, to show detail by increasing the scale, only a small portion of the diagram is included in the figure

Lines parallel to the left hand side of the triangle represent equal percentages of potassium citrate   Hence it is evident that in every case the salt content of the liquid is quite different from that of the total system and that of the solid phase   In no case are the tie lines parallel to the left hand side of the triangle

EXPERIMENTAL

The following experiments were carried out (a) with horse serum, (b) with serum from male rats, (c) from female rats, and (d) with human serum

The Results for Horse Serum

Six different samples of horse serum were obtained from the Cutter Laboratory, Berkeley   The blood was freshly drawn, defibrinated, the red blood corpuscles centrifuged off and the serum chilled immediately and kept at 0°   All six gave the same type of curve, and in two they coincided   Typical sets of analyses are given in Tables I and II

It will at once be noted that where the potassium citrates have gone fully into solution the analyses check admirably   Where the duplicates in the last column are different, it is seen that this corresponds

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solid phases separating from the mixtures   All the lines connecting phases in equilibrium that is the corresponding O and triangle must be straight and pass through the λ representing the total original composition of the system.

It is important to note that the angle at the corner representing H_2O may be changed to a right angle making a rectangular diagram where protein concentration is plotted against salt concentration and the scales of the ordinates and abscissas may be changed without altering this property of the tie lines and without losing the advantage of being able to plot all possible compositions of the entire system on such rectangular graphs

Any discontinuities or changes of direction of the solubility curve in the triangular graphs on any of these diagrams are not straight lines on other diagrams by other authors where composition is not expressed in total weight per cent Their significance, however, remains the same

TABLE I

Horse Serum   Precipitation with Potassium Citrate—0°C —pH 6 9

Total composition by weight		Solid phase by weight		Liquid phase by weight	
Potassium citrate	Protein	Potassium citrate	Protein	Potassium citrate	Protein
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
12 20	7 753			12 57	7 641
14 02	7 592			14 07	7 532
15 85	7 429			16 14	6 926
17 68	7 264			17 95	6 596
19 51	7 094			19 72	6 104
21 36	6 926	15 15	28 37	22 02	5 171
23 21	6 758	16 82	26 05	24 73	4 047
25 07	6 587	19 42	23 24	25 59	3 748
27 55	6 363	21 63	21 70	28 59	3 223
28 80	6 246	22 41	21 73	29 75	2 906
30 68	6 073	25 30	18 66	31 85	2 452
32 55	5 898	26 80	19 30	34 33	2 080
34 41	5 720	27 63	18 20	35 11	1 744
36 28	5 541	31 34	16 90	38 42	0 952
38 12	5 358			40 60	0 513
39 94	*5 172*	*36 90*	*12 66*	*43 10*	*0 0567*

TABLE II

Horse Serum   Precipitation with Potassium Citrate 0°C —pH 6 8

Total composition by weight		Solid phase by weight		Liquid phase by weight	
Potassium citrate	Protein	Potassium citrate	Protein	Potassium citrate	Protein
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
15 12	5 60			15 16	5 44
15 32	5 60			15 27	5 56
20 74	5 22	13 37	29 56	21 10	4 32
20 74	5 22	13 51	29 37	21 07	4 32
23 87	5 01	13 10	30 98	21 03	4 34
23 87	5 01	16 29	26 82	24 63	3 06
34 18	4 30	26 76	18 84	35 48	1 09
23 18	4 30	27 06	18 65	34 86	1 10
41 84	3 76	34 75	16 95	43 73	0 059
41 84	3 76	35 21	14 77	43 45	0 045

with the difference in the next to the last column, and upon plotting the data are found to lie upon the same equilibrium curve

Fig 1 gives the complete diagram for sample VI of horse serum which had been slightly concentrated by freezing   Some of the detail of this diagram is found in Fig 2
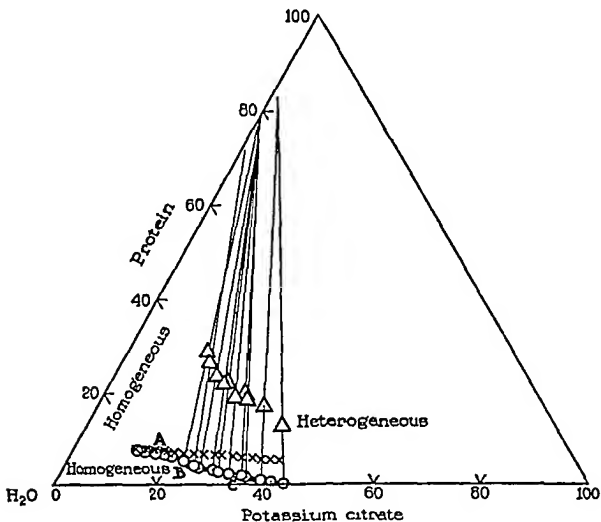
Fig 1 Horse serum, sample VI   Phase rule diagram at 0°C at pH 6 8
In Figs 1 2 and 4–6

  x = total composition
  O = liquid phase
  △ = solid phase

The most obvious feature of the phase rule diagrams is that the tie lines definitely do not point toward anhydrous protein but tend to converge at a protein containing between 21 and 25 per cent of H_2O and quite possibly some salt   For the general shape of the diagram reference should be made to the more elaborately annotated diagram

in the preliminary study of purified horse serum globulin and $(NH_4)_2SO_4$ [4]

The general nature of the equilibrium is surprisingly similar to the liquid boundaries found with soap solutions and gelatin

The discontinuities in all the solubility curves are taken up in the discussion
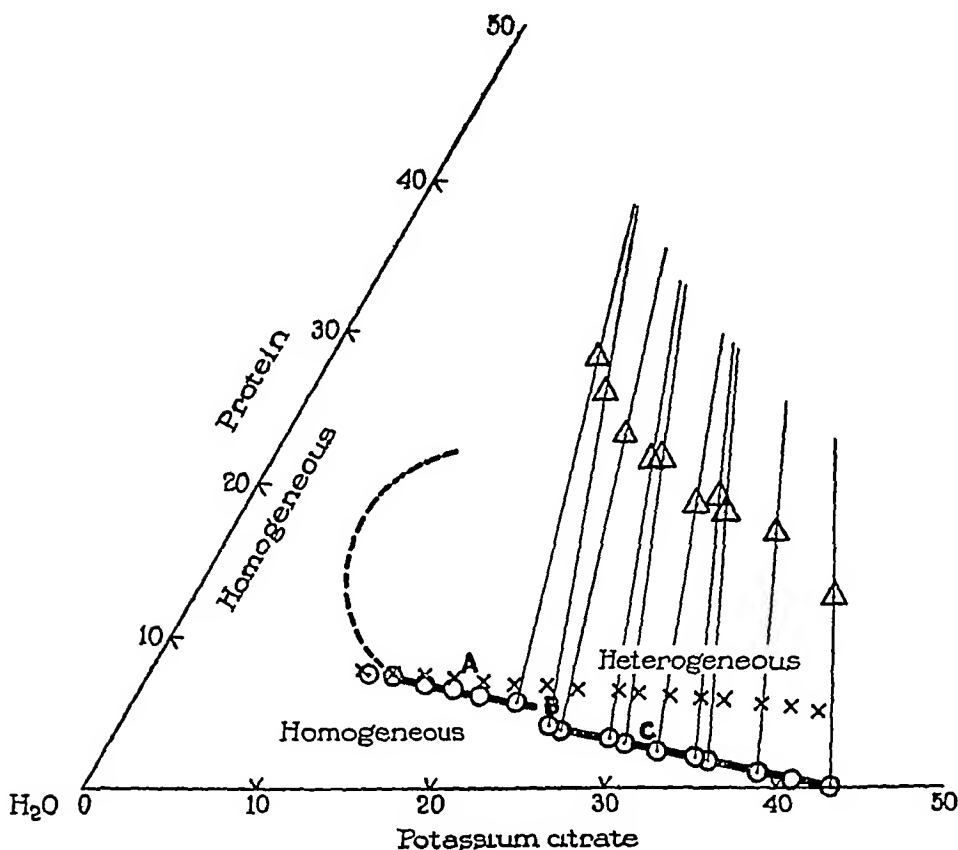
FIG 2 Horse serum, sample VI   Phase rule diagram at 0°C at pH 6 8

Results with Male and Female Rat Serum

The serum was obtained from pools of 30 or more rats of the same sex and age The blood was allowed to clot and the serum separated by centrifuging   A control experiment with horse serum showed no difference between serum prepared

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[4] McBain, J W , and Jameson, E , _Tr Faraday Soc_ , 1930, 26, 769

in the two manners The samples for precipitation were much small h
horse serum being from 2 to 15 gm instead of 40 gm and in one h
amount of solid phase separating from the smaller samples was not r
accurate analysis

sentative of three similar experiments, two with individuals and one
with a pool of serum from six persons   Figs 5 and 6 show the results
at pH 6 8 and pH 5 5 respectively
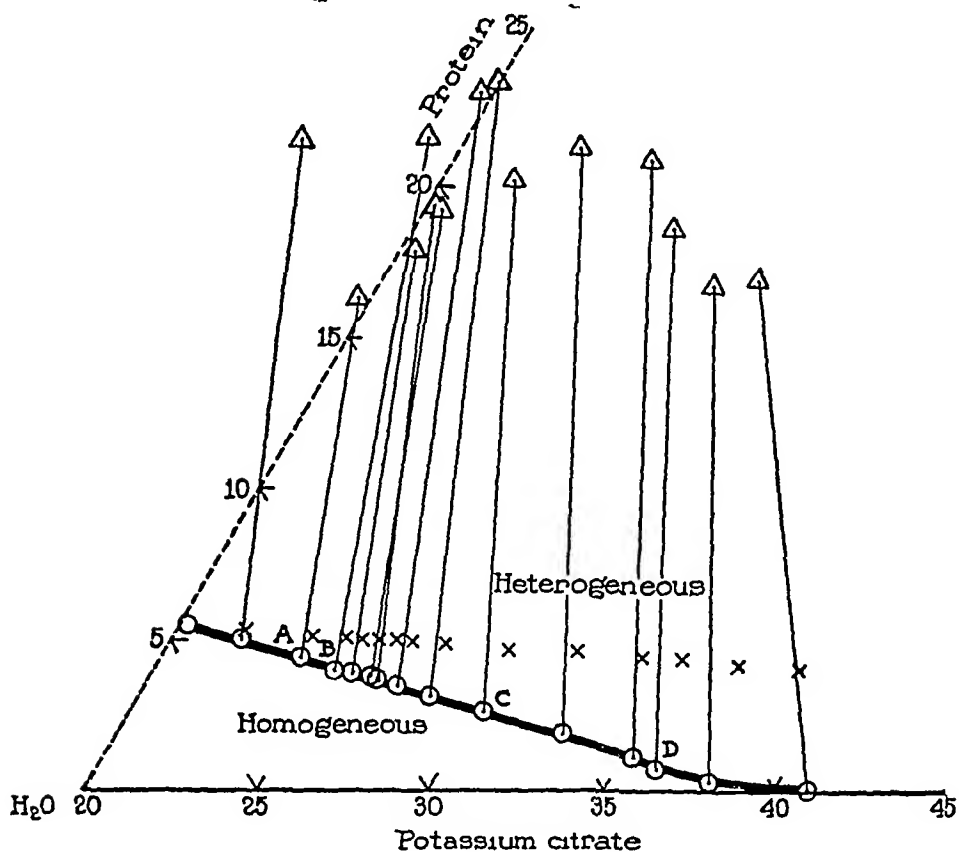
FIG 4 Diet — stock. Blood serum from 40 female rats   Phase rule diagram
at 0°C at pH 6 8

DISCUSSION

(a) There are four kinds of protein   As may be seen from Figs
1 to 6, the curves separating the homogeneous and heterogeneous
areas are of a similar type

The first section of the curve before the break at 22–23 per cent
salt with horse serum and 24–25 per cent with rat serum may be
assumed to represent an equilibrium of the liquid phase in contact
with a phase which is probably another liquid, protein A

The break which follows seems at first to be absent in human

serum and in female rat serum When the temperature or pH at which precipitation takes place is changed, this break may be made to

TABLE III

Stock Diet

Blood Serum from 40 Male Rats—100 Days Old   0°C —pH 6 8   Precipitation with Potassium Citrate

Total composition by weight		Liquid phase by weight	
Potassium citrate	Protein	Potassium citrate	Protein
Experiment 1			
per cent	per cent	per cent	per cent
19 61	5 412	19 85	5 314
23 58	5 135	24 03	4 529
25 57	4 997	25 84	3 883
26 57	4 925	27 22	3 605
27 56	4 857	27 84	3 616
29 54	4 718	30 17	2 881
31 51	4 580	32 19	2 371
32 49	4 510	33 69	1 765
34 46	4 372	36 02	1 088
35 43	4 301	37 10	0 734
37 36	4 160	39 54	0 1472
Experiment 2			
20 63	5 339	20 30	5 056
21 58	5 271	21 78	4 778
22 62	5 221	22 30	4 745
23 49	5 140	23 55	4 623
24 45	5 071	24 53	4 135
25 41	5 006	23 02	4 601
27 30	4 872	27 58	3 502
29 24	4 737	29 83	3 062
31 16	4 602	32 13	2 467
33 07	4 468	34 15	1 646
34 97	4 330	36 37	0 883
36 86	4 191	38 99	0 2051
37 81	4 122	39 78	0 0963

appear in the female rat serum [5] In Fig 6 showing human serum at pH of 5 5 in a small range of salt concentration, this break is seen to

[5] Unpublished

occur   This second protein phase, B, appears at about 23 per cent salt   The shape of the curve as it falls abruptly to the curve C has not been determined   The curve C again becomes a straight line showing equilibrium with a third phase, C, containing 20 to 25 per cent water and possibly some salt

The third change in direction and appearance of yet a different fourth solid phase, D, is obvious in human serum and in the rat serum

TABLE IV

Human Blood Serum from a Normal Person   Precipitation with Potassium Citrate—0°C —pH 6 8

Total composition by weight		Solid phase by weight		Liquid phase by weight	
Potassium citrate	Protein	Potassium citrate	Protein	Potassium citrate	Protein
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
17 94	6 422			18 14	6 252
19 93	6 264	13 78	28 7	20 22	5 814
21 91	6 101	17 32	21 0	22 27	5 284
22 90	6 021	17 54	23 85	23 26	5 157
23 89	5 941	21 15	20 55	24 30	4 934
24 88	5 862	20 22	20 65	25 41	4 655
25 87	5 782	22 16	18 7	26 43	4 601
27 85	5 622			28 61	4 138
29 82	5 462	23 04	19 2	30 51	3 674
31 78	5 302	25 70	17 4	32 29	3 335
32 76	5 220	20 33	18 5	33 39	2 976
33 74	5 141	28 36	15 95	34 73	2 491
35 68	4 978	30 81	17 0	36 51	1 726
37 61	4 815	32 90	19 1	39 82	0 558
39 52	4 652	33 43	20 3	43 30	0 014

(especially in the male) but not in any of the six horse serum samples It is evident from the work of others that this change does appear in horse serum after dilution and in the presence of $(NH_4)_2SO_4$ at room temperature

(*b*) There is a straight line relationship between the protein and salt concentration except where fraction D appears when it becomes a curve asymptotic to the base of the triangle

There is a rough proportionality between amount of protein in a

mixture and that remaining in solution, as may be shown by plotting all the horse serum curves on one diagram

One fraction does not cease to precipitate when another begins The question as to whether the fractions are individual proteins or not cannot be answered from these curves   The straight line relationship
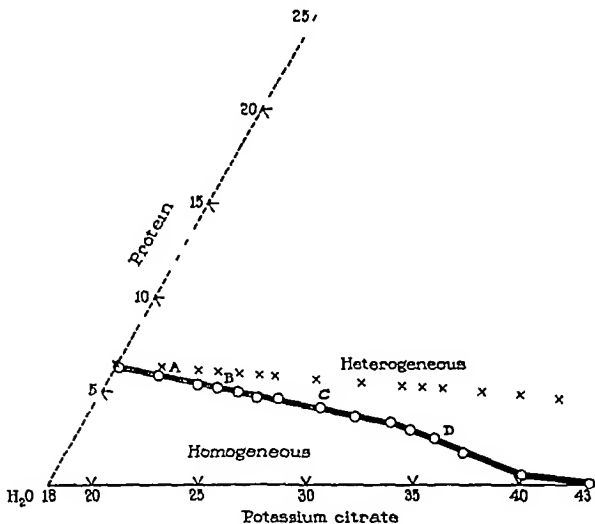
FIG 5 Human serum   Phase rule diagram at 0 C at pH 6 8

and continuous curve of D would indicate that they must either be single proteins or continuous series of compounds or solid solutions

The existence in the case of a rat serum of a high salt concentration where there is a definite solubility regardless of the amount of protein in the original mixture supports the view that the protein fractions are definite individuals [5]   However, it is probable that the high con centrations of the salt used may break existing labile bonds   Also

the salt concentration is so high that only the solubility of the D fraction is appreciable   Further work is necessary to definitely decide this question

(c) The convergence of the tie lines at the upper left side of the diagram indicates that all the solid protein phases contain from 20 to
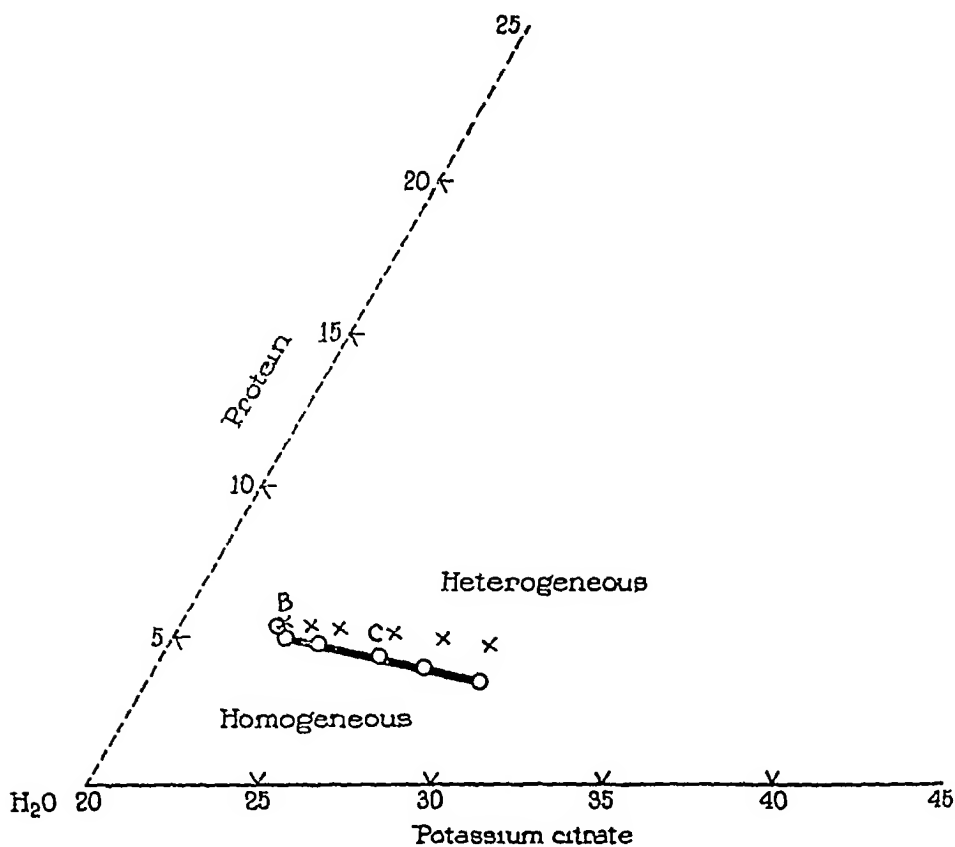
FIG 6 Human serum   Phase rule diagram at 0°C at pH 5 5

25 per cent of water   Consequently, they have a definite hydration even when separating from a wide range of concentrated salt solutions

(d) The salt concentrations at which the different protein fractions begin to be precipitated vary for different species   The relative per cents of the four fractions not only vary for different species but also for the two sexes

When plotted on a single diagram all of the solubility curves of a single species tend to converge at the same concentration of salt,

namely, about 45 per cent with horse serum, 38 per cent with rat serum, and approximately 40 per cent with human serum

SUMMARY AND CONCLUSIONS

There are four different kinds of protein in blood serum as shown by the solubility curves

They must be either single proteins, several continuous series of compounds, or solid solutions

The solid protein phases are hydrated

There are definite sex and species differences

Throughout the duration of this borderline study of the application of the phase rule to a physiological problem, I have been indebted to Dr T Addis and Dr J W McBain for helpful criticism

THE EFFECT OF HYDROGEN ION CONCENTRATION UPON THE INDUCTION OF POLARITY IN FUCUS EGGS

I Increased Hydrogen Ion Concentration and the Intensity of Mutual Inductions by Neighboring Eggs of Fucus furcatus[*]

By D. M. WHITAKER

(*From the School of Biological Sciences, Stanford University*)

INTRODUCTION

The egg of the marine brown alga *Fucus* is spherical   Many hours after fertilization (about 17 hours, in *Fucus furcatus* in thin cultures at 15°C in the dark (1)) a protuberance begins to bulge at one side of the egg, involving softening and extension of the cellulose cell wall   This protuberance extends, usually in the plane of the substrate, and at the completion of the first mitosis it is cut off by a cell plate as a more or less conical shaped cell (see Fig 1)   The first two cells are thus of very different shape, and their developmental fate or potentiality is entirely different   The protuberance cell extends and divides for some time in filamentous fashion and gives rise to the rhizoid of the new plant   The other cell gives rise to the thallus   Therefore, when the point of origin of the rhizoid protuberance is determined, the polarity and the whole developmental pattern of the embryo is determined

Several external agencies have been shown to determine the polarity of the *Fucus* egg   These are unilateral illumination (Kniep, Hurd, Whitaker (2–4)), electric current (Lund (5)), a pH gradient (Whitaker (6)), and the presence of near by neighboring eggs (Kniep, Hurd, Whitaker (2, 3, 7))   There are reasons for supposing that at least some of these actions resolve into a common underlying action, but

for the present purpose attention will be chiefly confined to the last, namely, the inductive effect of cells upon each other

Kniep (2), working on several species of *Fucus* on the Norwegian coast, reported that rhizoids tend to form on the sides of eggs toward neighbors and he credits Rosenvinge with having made the observation earlier   He found further that the rhizoids tend to form on the sides of eggs toward a near-by piece of adult thallus of the same or of another species of *Fucus*   Hurd (3) found that the tendency of rhizoids to form toward neighboring egg cells was so strong as to overcome the directive effect of light if the eggs were within two or three egg diameters distance   Whitaker (7) found that the directive effect of neighboring eggs was not specific and not dependent upon mitotic activity or growth in the directing cells, since unfertilized resting eggs of *Fucus vesiculosus* directed the developmental pattern of near-by fertilized eggs of *Fucus evenescens*   It was further found that two fertilized eggs alone in a dish did not develop rhizoids on the sides toward each other, but that an appreciable mass or group of eggs was necessary to act as a directing agency

Purpose

The purpose of these experiments has been to determine the relations of (a) egg concentration, and (b) increased hydrogen ion concentration, and their interrelationships, to the intensity of the mutual inductive effect

Method

Eggs of the hermaphroditic *Fucus furcatus f luxurians* have been used   Professor N L Gardner has been so kind as to identify living samples of the material The experiments were carried out on material collected at Moss Beach, about 20 miles south of the Golden Gate, in February and March, 1935   The material and its gametes were cared for and treated in the manner described in an earlier report (1)   In the present experiments the eggs were in the dark from before fertilization until the end of the experiments except for brief exposure to red light, which does not affect the phenomenon under consideration, and furthermore these exposures were confined to the period preceding 2 hours after fertilization, when the eggs have not yet become sensitive even to white light (4)   All work was carried out in a constant temperature room at $14\frac{3}{4} \pm \frac{1}{4}°C$

The media used were filtered normal sea water on the one hand, and filtered sea water acidified and buffered on the other   Measurements of pH were made

with a glass electrode The initial pH of the normal sea water ranged from 7 8 to 8 0 This of course does not represent the pH which came to occur at the surfaces of developing eggs where acid metabolites presumably lower the pH to an undetermined extent Supernatant sea water poured off egg cultures which had been reared 24 hours in the dark was always at least several tenths of a unit more acid than the initial sea water

Sea water was acidified with MacIlvaine s citric acid—secondary sodium phosphate buffer The buffer was made up by mixing 0 2 m phosphate and 0 1 m citric acid in such proportion as to give the desired pH A minimal amount of this was then added to the sea water to give what might be called marginal buffer capacity More specifically different amounts of buffer were added to sea water to find how much was necessary to hold a sample, in equilibrium with air constantly at pH 6 0 within about 0 1 unit for 24 hours even with cultures of eggs growing in the mixture It was found that 4 parts of buffer to 96 parts of sea water or 5 parts to 95 would just do this while if less buffer was used the pH would climb In the experiments 5 parts to 95 were used In this concentration the eggs develop perfectly This minimal buffer capacity was selected so that it did not necessarily preclude some degree of pH gradient about an egg or a group of eggs The buffer capacity was considerably greater than that of normal sea water, however The buffer sea water mixtures were clear between pH 5 0 and 6 4 Above 6 4 precipitation took place but no experiments were carried out in the precipitation range

After the results had been obtained it was thought advisable to repeat with another buffer system involving no phosphate or citrate ions to rule out the possibility that the effects were due to these rather than to hydrogen ions For this purpose a mixture of HCl and $NaHCO_3$ was added in sufficient amount to yield a buffer capacity which was empirically found to be approximately the same as that of 5 parts of MacIlvaine s buffer to 95 parts of sea water The mixture was equilibrated with atmospheric CO_2 by vigorous aeration with a heavy shower of minute air bubbles from a sintered glass filter This equilibration took about 2 days Triple glass distilled water was added to compensate for evaporation

Eggs were never placed in acidified media until 40 minutes after fertilization, to rule out any effects which acid might have on fertilization and entrance of the sperm All egg groups were arranged before an hour and forty minutes after fertilization

RESULTS AND DISCUSSION

An initial test of the acid tolerance of the *Fucus* eggs was made by rearing cultures in sea water acidified with citric acid–phosphate buffer They develop perfectly at pH 6 0, are retarded at 5 5, and at 5 0 practically no eggs develop although they do not cytolize In

FIG 1

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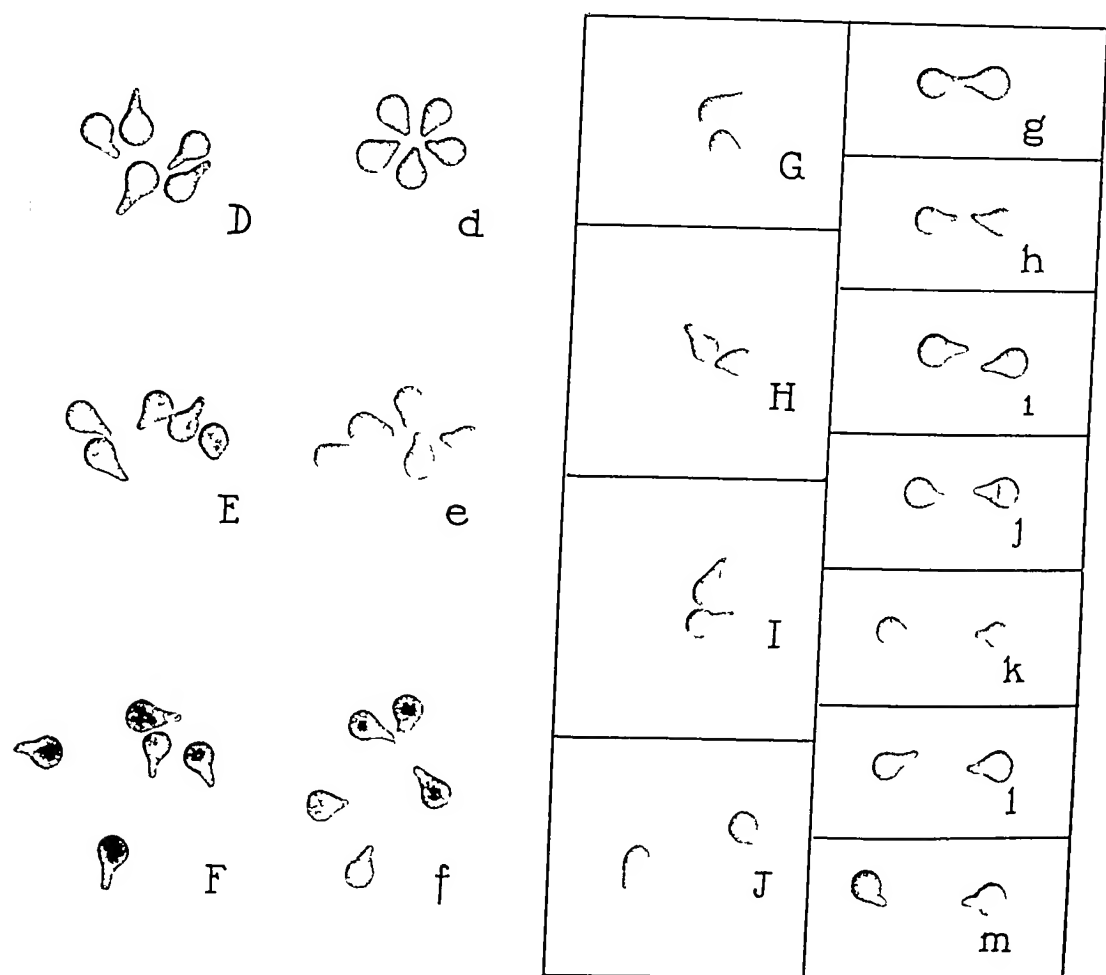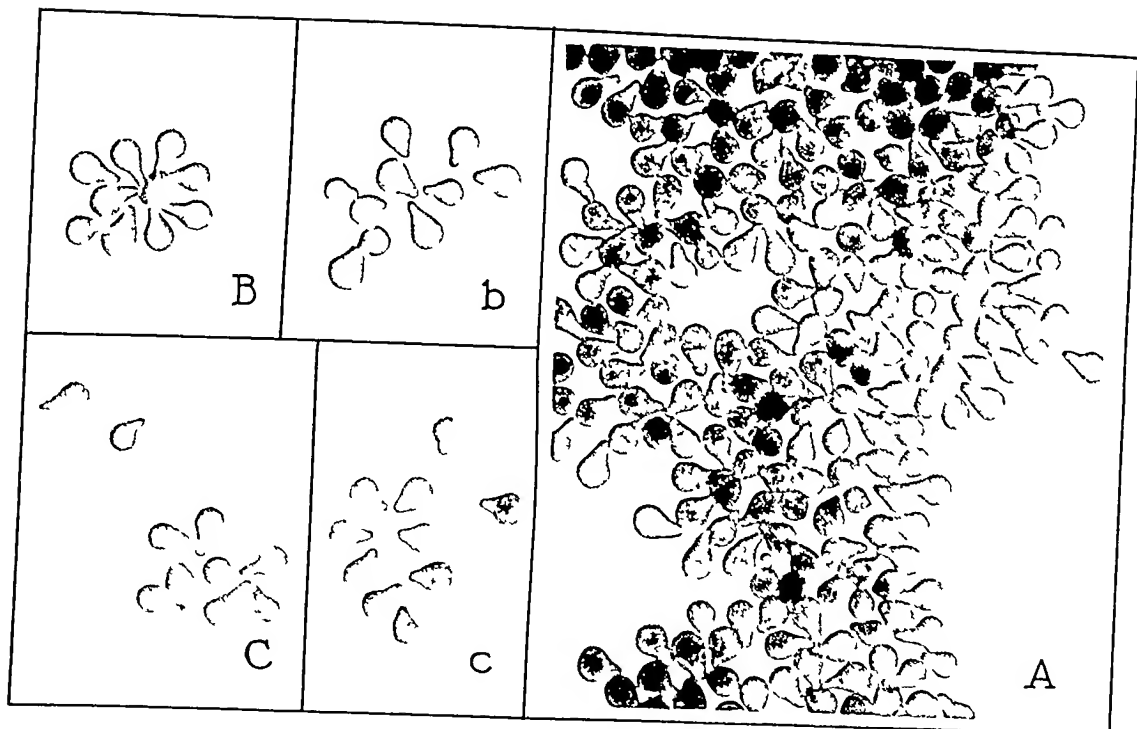
view of this result, the experiments to follow were all carried out at or very nearly at pH 6 0, with controls in normal sea water initially at pH 7 8–8 0

As has been noted, it was found several years ago (7) that eggs of *Fucus vesiculosus* do not develop rhizoids toward each other when there are only two eggs in a dish, but in larger masses of eggs the peripheral ones, having no neighbors on one side, develop rhizoids on the side which is in the resultant direction of the neighbors These observations have been repeated and confirmed on *Fucus furcatus*, and samples of the results are shown in Fig 1 A, G, H, I, J In Fig 1 all cultures marked with capital letters are in normal sea water Cultures marked with small letters are in acidified sea water at pH 6 0 Fig 1A shows a small sector of a large mass in which the same relations held throughout, among thousands of eggs Many other similar mass cultures showed the same thing It will be noticed that somewhat above and to the left of the center of Fig 1A there is a space free from eggs and that here as well the eggs lining the space have developed the rhizoids toward the resultant direction of neighbors (*i e*, pointing outward toward the mass in this case)

To find out how many eggs must be in a group in normal sea water to bring about the group effect, aggregations of different sizes, from two eggs up, were tested It soon became evident that groupings of two, five, ten eggs, and large masses covered the significant range for an answer, and a considerable number of each were made up and reared in the dark Comparable aggregations were arranged in sea water at pH 6 0 for comparison The aggregations of two and five eggs respectively were reared in small 1 cc Syracuse dishes in 1 cc of solution 5 mm deep No other eggs were in the dish The aggre

Fig 1 Photomicrographs of developing *Fucus* eggs in aggregations of different sizes Groups marked with capital letters are in normal sea water (initial pH 7 8–8 0) Groups marked with small letters are in sea water acidified to pH 6 0 In normal sea water rhizoids tend to form in resultant direction of neighbors only in larger groups of ten or more (A, note peripheral eggs, B C) and not in smaller groups of five or two (D, E F, G H, I J) In acidified sea water intensified mutual inductions occur not only in aggregations of ten (b c) or more but also when only five or two eggs are alone in a dish (d, e f, g, h 1 j k 1 m) See text

gations of ten eggs were in Petri dishes in the same depth of solution, and when there were several aggregations in the same dish they were at least 2 cm apart to avoid mutual influences   One exception to this is shown in Fig 1B which is an aggregation of ten in the same dish with the large mass A, as a result of which it shows an especially strong group effect

The distance between eggs in an aggregate is of course as important as the number of eggs, or more so if the inverse square law holds The distance factor will be considered in the simpler case of only *two eggs in a dish   An inspection of the typical cases shown in Fig 1* (capital letters) shows convincingly that in normal sea water the group effect operates in large masses, and at least to a considerable extent in aggregates as small as ten eggs, especially if the eggs are not far apart   It is not present in aggregates of five eggs or less   In sea water acidified to pH 6 0 on the other hand, the mutual inductions occur not only in large masses, but also (Fig 1, small letters) in aggregations of ten eggs more strikingly and over greater distances than in normal sea water, and in groups of five eggs, and even between two eggs alone in a dish provided they are not more than about 4 egg diameters[1] apart

To obtain a quantitative comparison, while also avoiding the complications of resolving components with variable egg distances in the patterns, some hundreds of eggs were set up in the weakest combination, namely with two eggs in a dish   Some were in normal sea water, others were in sea water acidified to pH 6 0 with citric acid—phosphate buffer, and still others were in sea water acidified to pH 6 0 with hydrochloric acid and sodium bicarbonate   The distance between eggs was 4 egg diameters or less   Typical results are shown in Fig 1 G, H, I, J (normal sea water) and g, h, i, j, k, l, m (acidified sea water)   Imaginary perpendiculars were erected to an imaginary line joining the centers of the two eggs, and the eggs were classified as having the rhizoid originate on the side of the egg either toward or away from the neighbor   Cases in which the rhizoid was within 10° of the line to the center of the other egg were also recorded   Results are shown in Table I   It is seen that in the medium acidified

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[1] The diameter of the eggs ranges from 65 to 90 microns

with citric acid–phosphate buffer 92 per cent of the rhizoids originate on the sides of the eggs toward the single neighbor, and a third of them do so within 10° Essentially the same result is found, slightly less marked, in the medium acidified with HCl–bicarbonate The controls in normal sea water on the other hand show no rhizoid attraction whatsoever between the eggs Less than a random 50 per cent of the rhizoids form on the side toward the neighbor It is clear that the group effect is very greatly intensified at pH 6 0

Eggs developing in thinly spaced cultures in normal sea water develop the rhizoid protuberance on the side of the egg, as viewed from above, but usually with some degree of downward component,

TABLE I

Results of Rearing Two Eggs Alone in a Dish, 4 Egg Diameters or Less Apart, in Normal Sea Water and in Sea Water Acidified to pH 6 0 by Two Different Buffer Systems

Medium	Total number of eggs	Rhizoids on sides of eggs away from neighbor	Rhizoids on sides of eggs toward neighbor	Rhizoids toward neighbor 0 to 10
		per cent	*per cent*	*per cent*
Normal sea water pH 7 8 to 8 0	133	59	41	5
Citric acid—sodium phosphate buffered sea water pH 6 0	97	8	92	33
Bicarbonate—HCl buffered sea water pH 6 0	104	12	88	26

i e , statistically the rhizoid protuberance develops somewhat below the equator In medium at pH 6 0 the origin of the rhizoid is shifted further downward quite strikingly, and its elongation continues downward in many cases until the cell body has been lifted up until it falls to one side This may be an increased if not a created geotropism, intensified by acidity just as the group effect is intensified Another possibility is that the acid sensitization has caused the egg to respond to the slight gradient of substances diffusing from itself which must be created by the block to diffusion at the bottom of the dish Eggs were reared at pH 6 0 with an agar gel substrate 1 5 mm thick, instead of glass, to lessen the block to diffusion beneath These eggs also developed rhizoids with strong downward components In

normal sea water, eggs in heavily crowded masses also show increased downward components of rhizoid origin and extension

Since lowering the pH of the medium intensifies the inductive influence among eggs, we have an adequate explanation of why, in normal sea water, the influence exists only among eggs in aggregates of appreciable size   These aggregates probably lower the pH of their immediate vicinity to an intensifying level by the production of CO_2 or other acid metabolites   It can further be supposed that after sufficient general lowering of the pH the developmental pattern is organized or induced by the residual pH gradients which follow the patterns of diffusion from the eggs of acid substances   It has been shown directly (6) that the rhizoid forms on the acid side when the egg is in an artificially established pH gradient

It should be noted that the mutual inductions have been intensified at lowered pH in spite of the fact that, due to the increased buffer capacity used in the acidified medium, the steepness of pH gradients following diffusion of acid substances from the eggs must be opposed and reduced, although not abolished   This fact, together with numerous others, some of which are even more suggestive, point to the possibility that one of the auxins (plant growth hormone) may be involved in the *Fucus* responses, and that its action may underlie or be the common means of action of a number of the effective agencies Since auxin is a dissociating acid which is physiologically active in the undissociated molecular form, increased hydrogen ion concentration increases the activity of auxin (8–10)   Gradients of hydrogen ion concentration and the general level of hydrogen ion concentration would therefore act in linkage with the action of auxin   There is at the present time no proof that *Fucus* eggs contain auxin, and it is not profitable to discuss its possible rôle pending the outcome of further experiments

Since acidifying sea water liberates free CO_2, there is always some danger of ascribing effects to increased acidity which might actually be due specifically to increased CO_2 tension   The guard against this of course consists in re-equilibrating with the atmosphere, causing the excess CO_2 to escape   This often takes surprisingly long in sea water so that it is difficult to completely rule out the possibility   The equi-

librations in the present instance were of long duration after very stable pH had been attained (0 1 pH unit change or less in 24 hours) especially in the carbonate runs, so that it appears quite improbable that the effects ascribed to hydrogen ions can be due to CO_2

SUMMARY AND CONCLUSIONS

1 The eggs of *Fucus furcatus* develop perfectly in sea water acidified to pH 6 0 They are retarded at pH 5 5 At pH 5 0 they do not develop, nor do they cytolize

2 In normal sea water in the dark at 15°C, eggs develop rhizoids on the sides in the resultant direction of a mass of neighboring eggs The polarity and the whole developmental pattern of the embryo is thereby induced[2] This inductive effect does not operate, however, unless the directing mass is an appreciable aggregation of cells (10 or more), or unless there are numerous other eggs in the dish A group of five eggs alone in a dish do not carry out mutual inductions Two eggs alone in a dish do not develop rhizoids toward each other

3 When the sea water is acidified to pH 6 0 all sizes of aggregations carry out mutual inductions Two eggs alone in a dish now develop rhizoids on the sides toward each other, provided they are not more than about 4 egg diameters apart

4 *Increased hydrogen ion concentration thus augments or intensifies the mutual inductive effect*

5 This may explain why only larger masses of eggs show inductions in normal sea water, since presumably the larger masses considerably increase the hydrogen ion concentration locally

6 The nature of the inductive action is discussed

7 In acidified sea water at pH 6 0, compared with normal sea water at pH 7 8-8 0, the rhizoids originate and extend with a strongly increased downward component The substrate then forces further extension or growth of the rhizoid to be in the plane of the substrate

The author is indebted to Mr Edward Lowrance for assistance in carrying out the experiments

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[2] It is possible, if not probable, that in the egg an earlier polarity exists which is superseded by the new induced polarity

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SOME REMARKS ON THE PHOTOSYNTHESIS OF GREEN PLANTS

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I

Of recent years there have been considerable contributions to the literature of the photosynthesis in plants One must mention here the papers of Franck (1) and Stoll (2), who developed the theory pro posed by Willstatter (3), the interesting considerations of van Niel and Müller (4), and the detailed discussions by Gaffron and Wohl (5) and Emerson (6)

The most important of recent experimental discoveries in this field is the "photosynthetic unit" of Emerson and Arnold (7)   These authors have shown that only a small fraction, about 1/2000, of the total amount of chlorophyll present takes part directly in the assimila tion process   It seems that the chlorophyll is entering into photo synthesis only in certain photosynthetic units containing a few hun dred molecules   Gaffron and Wohl (5) also concluded from the previous assimilation experiments of Willstätter and Stoll (8) that about 1000 chlorophyll molecules must cooperate to bring about the reduction of one molecule of CO_2, during the experimentally deter mined time of the "Blackman period."

Another important point is the rôle of iron in the assimilation of green plants   Although the need for iron in the photosynthesis of green plants has been known for a long time and the presence of iron in the chloroplasts was established by Moore (9) yet the rôle of iron has not been considered in the modern theories   It is not only that the plants grown in the absence of iron remain pale or colorless (chlo rosis) and therefore show a low photosynthetic activity due to their

low chlorophyll content, but also, as Willstatter and Stoll (8) have shown, in these chlorotic plants some other important part of the photosynthetic mechanism must be imperfectly developed (10) It is conceivable that iron salts catalyze the oxidation of the colorless protochlorophyll, from which, according to Noack and Kiessling (11), chlorophyll is formed Noack (12) has recently found a relatively large amount of ionic iron in the chloroplasts of different green plants This suggests that iron salts may take a direct part in photosynthesis It has been shown experimentally (13) that it is possible to reduce different fluorescent substances (e g chlorophyll) in the presence of ferrous salts by the action of light, which is absorbed by these substances in the visible region of the spectrum Similarly the ferrous salts may reduce the CO_2 in the plants under the action of sunlight, a point which will be discussed more fully later

<center>II</center>

It is well known that the assimilation process of the green plants can be represented by the following stoichiometric equation

$$CO_2 + H_2O = \tfrac{1}{6}C_6H_{12}O_6 + O_2 - 112 \; K \, cal$$
<center>glucose</center>
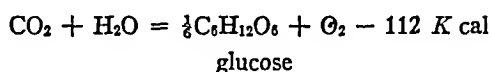
Warburg (14) has shown experimentally that 4 quanta must be absorbed for the reduction of one molecule of CO_2 In the case of red light ($\lambda \sim 660$ mμ) this corresponds to about 168 K cal (per mol), i e , with the small reserve energy of about 50 K cal the plant is able to provide for the activation energies of all the processes in the hydrogenation of the CO_2 Every individual process involved must possess a high quantum efficiency and a small heat of activation, and the intermediate products must have a long lifetime The last two conditions will be fulfilled with radicals as intermediate products and the high quantum efficiency is also compatible with this assumption, if the light reaction is followed by a sufficiently fast thermal reaction

Willstatter and Stoll (8) have shown experimentally that a complex between chlorophyll and CO_2 is easily formed in the presence of water It is possible that the Fe^{++} ions present are able to reduce a chlorophyll-CO_2-complex in the plant under the influence of sunlight

The decomposition of water into H and OH in the assimilation

process has previously been assumed by van Niel and others (4) However, the direct splitting of H_2O with a red quantum of energy ($\sim 50\ K$ cal /mol) is impossible because of the high binding energy (115 K cal /mol) of the H—OH bond This can only be accomplished with the help of Fe^{++} (or possibly another reducing agent) by sensitization with a substance which absorbs in the red region of the spectrum, as has been proved experimentally (16) The hydrogen formed hydrogenates the sensitizing substance (The direct photochemical decomposition under the influence of Fe^{++} with the accompanying formation of hydrogen gas requires an energy of at least 90 K cal ($\lambda \sim 2900$ Å) (17))

In this connection the behavior of the green sulfur bacteria is of particular interest According to van Niel and Muller (4) no catalase and most probably no iron are present in these bacteria Consequently they should not be able to reduce (assimilate) CO_2 with H_2O alone Actually they are only enabled to assimilate in the presence of H_2S which, in the course of the process is oxidized to elementary sulfur In view of the much smaller binding energy of the S—H bond ($\sim 88\ K$ cal) one realizes how these bacteria can produce H atoms from H_2S (even in the near infrared), by using the quanta absorbed by the sensitizing bacterio chlorophyll present These conclusions are in good agreement with recent investigations on the photochemistry of SH compounds in solution (18)

III

It seems at first very difficult to understand how the plant accumulates the 4 quanta necessary to reduce one molecule of CO_2 with practically no loss of energy It is easy to show by a simple statistical calculation that if only those molecules of chlorophyll, which under the given conditions have absorbed at least 4 quanta of light, can take an active part in the assimilation, the quantum efficiency would be very small indeed The quantum efficiency is, however, a relatively high one (14)

An explanation of this complete utilization of the absorbed energy is to be found in the peculiar structure of the chloroplasts and in the state of the chlorophyll in the living plastids According to the investigations of Liebaldt (19) and Mencke (20) the chloroplast, which

is optically anisotropic, consists of a lipoid phase, in which the chlorophyll is dissolved, which is itself dispersed in an aqueous "hydroid phase "

It seems that the chlorophyll has to fulfill two different functions, depending on its situation in the chloroplasts   The chlorophyll molecules on the surface of the lipoid phase (in contact with an aqueous phase containing Fe^{++}) combine with CO_2 to form a light absorbing chlorophyll-CO_2-complex and in this way take part in the reduction of the CO_2

The greater part of the chlorophyll molecules is dissolved in the interior of the lipoid phase and absorbs the energy of the light which is then stored in the form of electronic excitation energy   It is well known that electronic excitation energy is practically never directly transferred into kinetic energy (heat) (21)   Therefore the quanta absorbed in the interior of the lipoid phase will be handed over from one chlorophyll molecule to another by a sort of resonance effect and eventually reach the chlorophyll molecules on the surface   In this way all the energy absorbed in the interior can ultimately be used for the assimilation process on the surface   This process implies that the chlorophyll molecules in the lipoid phase are in a state of strong mutual interaction   The observed shift of the absorption maximum of chlorophyll in the living plastids by 150–200 Å   towards the red region as compared with chlorophyll in solution or in the colloidal state (22) may be due to interaction forces of this kind

The above considerations require that the lifetime of the excited chlorophyll molecule in the lipoid phase shall be of the same *order* of magnitude as the average time of reaction necessary for the complete reduction of one CO_2 molecule (the so called Blackman period)   The lifetime of the excited chlorophyll molecule in solution is about 0 01 second at 25°C (Kautsky (23)) and the Blackman period was found to be 0 02 second at 25°C (Emerson (29))   Both the lifetime of excited chlorophyll and the Blackman period increase considerably with falling temperature   It is also possible to explain Kautsky's observation that strongly assimilating leaves show a considerably weaker fluorescence than in the normal state (24)   In the case of non-assimilating leaves the energy coming from the interior of the lipoid phase is not captured on the surface and is eventually given up

as fluorescent light   On the basis of these arguments the photosyn
thetic unit of Emerson and Arnold is determined by the ratio surface/
volume of the lipoid globules in the plastid

$$\frac{\text{(Active) chlorophyll in the surface of the lipoid phase}}{\text{Chlorophyll dissolved in the interior of the lipoid phase}}$$

The obvious implication is that for every chlorophyll molecule on
the surface (actively reducing CO_2) there are about 500 molecules in
the interior which provide it with the necessary 4 quanta

This assumption does not seem to be in contradiction to the recent
considerations of Kohn (25), which on the other hand are unable to
explain the accumulation of energy

With regard to the size of such a structure, there seems to be no par
ticular restriction, as may be clear from the following remarks

Assuming, for example that the globules (in the chloroplast) are
small spheres (radius r), the chlorophyll molecule can be regarded (to
a first approximation) as a cylinder, constituted by the porphine
ring as the base of about 100Å^2 (radius ρ) and the phytol chain lying
on top of it, the height of the cylinder being about $h \sim 3\text{Å}$ These
dimensions are taken from measurements on surface films by Gorter
(26) and A Hughes (27)

We then have for the ratio

$$\frac{\text{Surface of the sphere} \times \alpha_s}{\text{Volume of the sphere} \times \alpha} = \frac{\text{(porphine) surface of one chlorophyll molecule}}{\text{volume of 500 chlorophyll molecules}}$$

where α denotes the fraction of the (total) surface occupied by the
chlorophyll and α_s denotes the fraction of the (total) volume occupied
by the chlorophyll

From the above equation we obtain

$$\frac{4\pi r^2 \times \alpha}{\frac{4}{3}\pi r^3 \times \alpha} = \frac{\rho^2 r}{500 \, \rho^2 \pi \, h}$$

$$r = 3\,500\,3\,10^{-8}\frac{\alpha_s}{\alpha}\text{ cm} \sim 4\,5\,10^{-8}\frac{\alpha_s}{\alpha}\text{ cm}$$

We do not know the value of the ratio $\frac{\alpha_s}{\alpha}$, but even assuming this
to be about unity, we obtain for the radius of the little sphere repre

senting the structural unit $r \sim 0, 4\mu$, which is of the order of $1/10$ to $1/20$ of the radius of a plastid   It is possible that the dark globules shown by photographs of chloroplasts in red light (28) represent this photosynthetic unit, the size of these globules being of the same order of magnitude as suggested above

<p style="text-align:center">IV</p>

The processes discussed above can be represented by a system of simple reactions showing the step-wise reduction of CO_2 to the CH_2O stage   (The asterisks in the following equations denote excited forms of molecules)

(Complex formation)
    (a) Chlorophyll (surface) $+ CO_2 + H_2O \rightarrow$ Chl—CO_2-complex
(Light absorption)
    (b) Chl—$CO_2 + h\nu \rightarrow$ Chl—CO_2^* (b') Chl—$CO_2^* \rightarrow$ Chl—$CO_2 + h\nu$
1 *(Reduction step)* Chl—$CO_2^* + Fe^{++} \rightarrow$ Chl—$CO_2^- + Fe^{+++}$, Chl—$CO_2^- + H^+ \rightarrow$
    Chl—COOH $2COOH \rightarrow H_2C_2O_4$ oxalic acid (by product)
    (Light absorption)
    (c) Chlorophyll (lipoid phase) $+ h\nu \rightarrow$ Chl* (c') Chl* $\rightarrow$ Chl $+ h\nu$
    (d) *(Energy transfer)* Chl—COOH + Chl* $\rightarrow$ Chl—COOH* + Chl
2 *(Reduction step)* Chl—COOH* + $H_2O \rightarrow$ Chl—H COOH + OH
    (e) *(Energy transfer)* Chl—H COOH + Chl* $\rightarrow$ Chl—H COOH* + Chl
3 *(Reduction step)* Chl—H COOH + $Fe^{++} \rightarrow$ Chl—H COOH$^-$ + Fe^{+++},   Chl—H
    COOH$^-$ + $H^+ \rightarrow$ Chl HC $= O + H_2O$
    (f) *(Energy transfer)* Chl—HCO + Chl* $\rightarrow$ Chl—HCO* + Chl
4 *(Reduction step)* Chl—HCO* + $H_2O \rightarrow$ Chl—H CHO + OH

Reactions analogous to (1) and (3) have been observed experimentally, therefore the energy relations need not be considered in detail here   For reactions (2) and (4) the total energy, Q, is given by $Q = Nh\nu + E_{C-H} - D_{H_2O}$ where D_{H_2O} (115 K cal) is the energy (per mol) required to split H_2O into H and OH, $Nh\nu$ ($\sim 42 K$ cal) the excitation energy of chlorophyll and E_{C-H} ($\sim 94 K$ cal) the energy of the C—H bond   We therefore have $Q \sim 25 K$ cal , so that both reactions (2) and (4) can be regarded as sufficiently exothermal

This scheme is but one of several alternative representations of the theory   In this case all processes, except reaction (1), are taking place during the Blackman period   The formation of oxygen is considered to take place with the help of catalase from hydrogen peroxide formed according to $2OH = H_2O_2$ Two OH radicals are formed

directly in reaction (2) and (4), whereas two additional OH radicals must be formed in the reduction of Fe^{+++} formed in reaction (1) and (3) $2H_2O_2$ yield finally 1 mol O_2 One might assume that the Fe^{+++} ions formed in the assimilation process are reduced to Fe^{++} in the course of the respiration process and that the augmented respiration is thus connected with assimilation (30) It is possible that the carotenoids of the chloroplast also enter into this process For the stationary state and constant illumination one can derive some conclusions with regard to the kinetics which are in good agreement with experiment (31)

(α) For small light intensities, when the rate of formation of the Chl—CO_2-complex (reaction a) is fast with respect to the subsequent reactions, the rate of assimilation is determined solely by the amount of light absorbed per second (I_{abs}) and therefore the temperature coefficient in this region must be unity For complete absorption (rate $\sim I_{abs} \sim I$) the rate varies linearly with the intensity of the incident light (I_o) For small absorption (rate $\sim I_{abs} \sim I_o$ [Chl] [CO_2]) the rate is also a linear function of the [Chl] and [CO_2] concentrations in the stationary state

(β) For high light intensities the formation of the complex (Reaction a) is the time determining factor In the case of sufficiently small CO_2 concentrations this can also occur at smaller light intensities The rate is then given by rate $\sim$ const $e^{-Q/RT}$ [Chl] [CO_2], where Q is the heat of activation of reaction (a) In this case the rate is independent of the light intensity, in agreement with the experiments of Blackman (34), Warburg (14), and others The dependence on temperature is given by log rate $\sim -\dfrac{Q}{RT}$, in agreement with the experiments of Emerson (33) Using intermittent light the kinetics are in some respects more complicated, because we cannot assume stationary conditions In fact, the time required to establish a stationary state is of the same order as the light period

The above discussion could readily be extended to provide some other conclusions which, however, for the present seem to be of little interest

SUMMARY

1 It is suggested that in the assimilation process of green plants the reduction of the CO_2 takes place with the help of Fe^{++} ions (present in the chloroplast) under the influence of light, which is absorbed by a sensitizing chlorophyll-CO_2-complex

2 It seems that the chlorophyll has to fulfill two different functions depending on its situation in the chloroplast   The chlorophyll molecules on the surface of the lipoid phase (in contact with an aqueous phase containing Fe^{++}) combine with CO_2 to form a light absorbing chlorophyll-CO_2-complex and in this way take part in the reduction of the CO_2

The light energy is also absorbed by the greater portion of the chlorophyll, which is dissolved in the interior of the lipoid phase, and eventually handed over to the chlorophyll molecules on the surface

3 The photosynthetic unit of Emerson and Arnold may be determined by the ratio

$$\frac{\text{(Active) chlorophyll in the surface of the lipoid phase}}{\text{Chlorophyll dissolved in the interior of the lipoid phase}}$$

so that for every chlorophyll molecule on the surface there are about 500 molecules in the interior, which provide it with the necessary quanta

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FLICKER AND THE REACTIONS OF BEES TO FLOWERS

BY ERNST WOLF AND GERTRUD ZERRAHN WOLF

(*From the Biological Laboratories, Harvard University, Cambridge*)

The quantitative evidence secured thus far about the visual functions of the eye of the honey bee is based upon the fact that a bee will respond to a displacement of a pattern within its visual field Whether we are dealing with visual acuity tests (Hecht and Wolf (1928-29)), intensity discrimination (Wolf (1932-33 a,b)), dark adaptation (Wolf and Zerrahn Wolf (1935-36)), or marginal recognition of flicker (Wolf (1933-34)), the reaction of the bee always depends upon the transition of visual elements from one state of excitation into another Even when confronting a bee with the task of distinguishing patterns of different design and coarseness the same is true If bees are to choose between a series of patterns of the same area but different in design, the number of choices of each pattern is proportional to the lengths of the contours within each pattern (Zerrahn (1933)) This again indicates that the transitory stimulation produced during the bee's flight over the patterns by reason of the edges separating black from white, arouses the response A different proof for this kind of reaction to patterns can be given by conditioning bees to flickering fields of equal size but different flicker frequencies Under such conditions the bee's choices are directly proportional to the flicker frequencies, provided the frequencies are below fusion (Wolf (1933)) At the same time we have evidence that only the numbers of alternating stimuli reaching the ommatidia are responsible for a choice, since patterns different in area and coarseness of design can be combined in such a way that equal numbers of choices are obtained (the number of elements stimulated by transition being equal for the patterns presented for choice) (Wolf and Zerrahn Wolf (1934-35)) On the other hand, two flickering fields will have the same stimulating effect upon bees if for both the product of flicker frequency × area is the same (Wolf and Zerrahn Wolf (1934-35))

We therefore come to the conclusion that recognition and distinction of patterns by the bee is based upon the transitory stimulation produced by a pattern and that the pattern as such is of no importance This theory is in disagreement with the experimental evidence and the conclusions of Hertz (1930, 1931, 1933, 1934 *a,b,c*, 1935), who claims that pattern discrimination by the bee is based upon recognition of the pattern as such, upon the distinction of patterns as patterns, and a memory of form

If we recall the fact that the bee's visual acuity is only about 1/100 of ours, its intensity discrimination only 1/25, we hardly can assume that the bee's vision is *a priori* sufficient for any higher visual perception such as distinction of patterns and forms   In only one respect is the function of the bee's eye equal to the human, namely in the recognition of flicker   For the human eye the maximum critical flicker frequency is between 45 and 53 per second (Hecht, Shlaer, and Verrijp (1933–34))   At very high illuminations the bee will still respond to 55 flickers per second, so that its recognition of flicker is certainly as good as in man, if not even better   It seems generally true that flicker perception in Arthropods is higher than in mammals So far we know that dragon fly larvae can react to slightly over 60 flickers per second (Salzle (1932), Crozier, Wolf, and Zerrahn-Wolf (1936–37)), and *Eupagurus* to 56 flickers per second (Brocker (1935))

It seems probable therefore that flicker may play an important rôle in the vision of the bee, and the question arises whether reactions to intermittent stimulation might play a part in nature   It was pointed out in earlier papers (Wolf (1933, 1935)) that perhaps flickering effects produced by the relative motion of the bees and flowers while collecting nectar might have an effect upon the bees' settling on the flowers   The finding of small blossoms closely spaced, even if their particulars of flower formation are below threshold visibility, might be facilitated by the relative motion of the flowers in regard to the bee's eye, and thus through the production of flicker cause the bee's reaction to the flower

We had made the observation that the number of bees settling on particular flower beds on quiet and on windy days is greater when the flowers are slightly moved by the air   There is no question that with an air current odors from the flowers might drift over a greater area

and thus attract more bees It can be shown, however, that the intermittent optical stimulation has a considerable effect upon the bee's reaction when odors are excluded

By studying the bee's reaction to natural and artificial flower beds which can be moved, evidence can be brought about for the effect of flicker upon the bee's reaction toward the experimental arrangement

FIG 1 Arrangement of 25 flower pots planted with *Phlox sabulata alba* Any one of the pots can be put on a turntable and rotated By the increase in flicker produced by the rotation more bees are attracted to the moving flowers

For test purposes bees from one of our colonies are conditioned to collect a sugar solution from Petri dishes about 50 meters from the hive The dishes are placed in between an arrangement of 5 x 5 flower pots making a flower bed one square meter in size in which there are planted *Phlox sabulata alba*, a flower which has no particular odor very small white blossoms and which is rarely visited by bees After a constant stream of bees visits our flower bed and settles with certainty on the dishes which are frequently shifted around among the pots so as to avoid conditioning to a particular location in the flower bed the dishes are removed for test and the bees observed The bees search over the flowers and will gradually settle on the flowers in bunches in the manner observed when bees spontaneously or after conditioning react to patterns The arrangement of the

flower pots is such that each one can be placed on a turntable and can thus be rotated at a slow speed   A turntable is attached to a kymograph motor which has been adjusted in such a way that $\frac{1}{2}$ to 1 rotation is obtained per second   We therefore have the possibility of increasing the intermittent stimulation produced by the flower bed by rotating one pot   Under these conditions the bees do not settle at various places over the whole field but follow the rotating pot in a circling cloud   Unfortunately the bees do not settle down on the flowers, but gradually disappear and return to the hive   It would be of great importance to obtain some quantitative measure for the bee's reaction   We therefore changed the

Fig 2   Artificial flowers arranged in flower pots   Each pot can be rotated to increase the flicker effect produced by the flowers

arrangement by covering the whole bed with a glass plate on which the bees are fed   This arrangement has great advantages since the glass plate can be washed before test so that all traces of odors which might cause reactions are removed, and the food can be spread over the whole surface of the glass plate so as to avoid conditioning to a certain place   Then under these conditions, after removing the food and cleaning the covering glass plate, one of the flower pots is rotated A slight reaction to the rotating section can be observed, but it is not as clear as in the previous case   It seemed that the reflection of light from the glass greatly disturbed the bees and that possibly the distance of the flowers from the bee's eye reduced the visibility to a degree which interfered with a proper reaction

In another test we employed artificial flowers instead of the small white blossoms of *Phlox*. The flowers were cut out of white cardboard 7 cm in diameter and with 6 flower leaves leaving spaces of about 8 mm between them at the margin. These artificial flowers were fixed on top of thin wooden sticks and 3 of them were placed in each of 16 flower pots. The whole arrangement was covered with a glass plate on which the bees were fed. One of the pots can be rotated. During test when the food is removed and a section of 3 blossoms is in motion the bees during their search for food react to the motion, but again the precision of the reaction is not very great. Much better results are obtained if during test
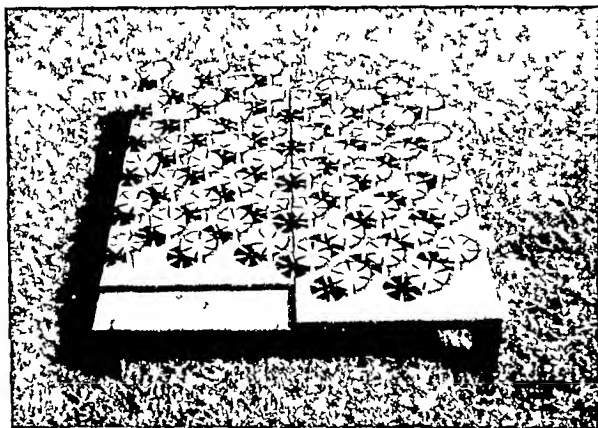
FIG 3. Artificial flower bed on which bees are fed. One half can be moved back and forth. By the increase in flicker produced by the motion the number of bees visiting the moving section is increased.

the glass plate is removed. The bees then follow the rotation in a cloud and occasionally settle down. The reaction in general is clearer than with the *Phlox* but is not yet satisfactory. It seemed that for clear results a close association between flowers and food was desirable.
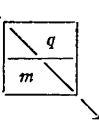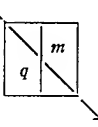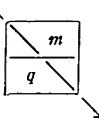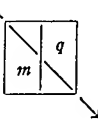
In a further experiment we made use of an artificial flower bed 1 × 1 meter in size on which there were placed 10 rows of artificial flowers evenly spaced. The flower bed is divided into two halves one of which rests on rails so that it can be moved forward and backward over a length of 20 cm. The speed of movement is such that a forward motion takes 1 second then the section stays quiet for

1 second, then the backward motion takes 1 second again   The flowers on the bed consist of glass tubes 6 cm high, 8 mm in diameter, around the upper end of which the flower leaves (7 cm in diameter) are fitted   The flowers are set into holes in the base of the bed so that they stay rigid during the motion   Each tube is filled two thirds full with paraffin so as to prevent the collecting bees from creeping too deeply into the tubes   For conditioning the bees all the flowers are filled with a sugar solution   The bees which come to the bed spread rather evenly over the whole surface of the bed and take the sugar solution   The bees show a tendency to cross over the field in a certain direction, which is due to the location of the hive and the easiest way of access to the feeding place   If,

TABLE I

Data on the visits of bees in a 5 minute period to a flower bed consisting of two sections, one of which stays quiet (q) while the other is moving (m)   In the top row the relative positions of the quiet and the moving sections are shown The arrow indicates the direction in which the bees from the hive approach the artificial flower bed

No of test	m	q	m	q	m	q	m	q
1	129	80	123	73	130	73	110	77
2	142	67	119	69	106	69	107	67
3	160	89	173	87	194	81	175	89
4	158	102	139	84	133	60	160	86
5	164	97	189	83	168	98	176	97
Total	753	435	743	396	731	381	728	416
Mean	151	87	149	79	146	76	146	83
Ratio	1 7 1		1 9 1		1 9 1		1 8 1	

however, one of the flowers is occupied by one or two bees, the newcomer goes on searching until it finds a proper place for feeding   The evenness of the distribution permits a quantitative study of the bees collecting if one section of the flower bed is moved

For test we filled all tubes up to the margin with sugar solution   During this process the bees are kept away from the flowers   We then set one half of the bed into constant motion for 5 minutes   During that time the bees settling on either half are counted, one observer watching the moving section, the other the quiet one   Since we observed that there was a certain direction in which the bees crossed the field, for each new test we turned our arrangement through 90°, so

that the bees would meet the quiet and moving parts at different angles   By going through the whole cycle five times a picture of the bee's reactions can be given (Table I)

The data show that without doubt the number of bees is greater on the moving half of the flower bed compared with the quiet half   The ratios are almost 2 1 One might expect that the frequency of visits to the moving section should even be higher, on account of the flicker effect   If we consider, however, that the moving section as a whole is shifted and thus the distance between the flowers stays the same the flicker effect is not very great   One should expect a greater number of bees to collect on the moving flowers if one could introduce an irregularity of motion such that each row of flowers is moved independently   Since this procedure would involve great technical difficulties, we have to rely on the data as they are presented here   There is one striking fact which permits the assumption that the relative shift of the flowers within a certain area of the field induces more bees to settle down   The two rows at the dividing line between the quiet and moving sections are more frequently visited than the rest of our arrangement, and the row on the moving and on the quiet part obtain about equal numbers of bees   Due to this fact a great many bees are counted in favor of the quiet section, even while their reaction has been induced by the shifting of the flowers, and thus the observed ratio of visits to the two halves of the field is not greater than 2 1

With these results it seems justifiable to assume that flicker plays a rôle in nature while bees are visiting flowers   With a reaction to flicker the finding of flowers by the bee can be understood easily, whereas the low visual acuity and intensity discrimination could not support an adequate interpretation (Wolf (1933, 1935))

SUMMARY

Bees were conditioned to collect food on natural and artificial flower beds, parts of which could be set into rotation or side to side movement   Through the relative motion of the flowers the number of alternating stimuli upon the bee's eye is increased   Due to the fact that bees show a strong reaction to intermittent optical stimulation, the proportion of bees settling on the moving section of the flower bed is increased   It seems probable therefore that the visual reaction of bees to flowers in nature is largely due to the flicker effect produced through the motion of the bees relative to the flowers

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THE EFFECT OF ORGANIC IONS ON THE MEMBRANE POTENTIAL OF NERVES

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The phenomenon of the injury potential of nerve and muscle is generally explained by the assumption of a membrane surrounding the individual cell and separating two different electrolyte solutions inside and outside of the cell   The potential, then, is due to the particular properties of the membrane, and to the fact that the ionic composition of the cell content differs widely from that of the inter cellular fluid

As to the first point, different views are held   (1) The membrane acts as a molecular sieve (Ostwald (1890), Bernstein (1902), Höber (1905), Michaelis (1926), Netter (1928)) and the potential is essen tially a diffusion potential   (2) The membrane is a homogeneous non aqueous layer and the potential is a phase boundary potential (Beutner (1920))   (3) The membrane is a homogeneous non aqueous layer and the potential is a diffusion potential (Osterhout (1933))

The purpose of the present paper is not primarily to furnish evidence for one of these views   Part of its results are, however, best under stood, as will be shown, by the assumption of a porous structure Also for reasons discussed in another paper we prefer the first inter pretation, assuming that an oriented molecular structure rather than a homogeneous phase constitutes the membrane   Investigations on x ray patterns of nerves, in which definite interferences could be attributed to the myelin sheath (Boehm (1933)), seem to justify this assumption, at least for the myelin sheath

As to the second point, several workers have shown that the ionic content of the nerve fibers is very different from the intercellular

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fluid, especially with respect to potassium Since this ion has a particular effect on the injury potential of muscle and nerve the assumption was made that the potential is mainly due to the unequal distribution of potassium

Recent work, however, seems to indicate that this view is incomplete A connection between metabolism and nerve potential was suggested by the work of Gerard (1929) and Furusawa (1930), who found a reversible decrease of the injury potential in absence of oxygen Also the after potentials during activity seem to be connected with the metabolism of the nerve (Levin (1927), Gerard (1929), Amberson and Downing (1929–30), Furusawa (1930), Gerard (1930), Amberson, Parpart, and Sanders (1931))   It may be argued that the membrane itself is injured by the asphyxia, losing temporarily its characteristic properties, in terms of the theory of Bernstein and Hober, its selective cation permeability or its selective potassium permeability   It is, however, hard to explain how the effect can be reversible, since no force seems to be present to drive back the potassium which left the cell during the time of increased permeability

Some dynamic factor, related to the metabolic activity of the cell, seems to be lacking in the present picture of the potential mechanism A different kind of connection may be suggested, therefore   The metabolism furnishes ionized metabolites which, without necessarily affecting the membrane, act on the potential   The recent excellent work of Teorell may be mentioned here   He has worked out both theoretically and experimentally a more general type of Donnan equilibrium, in which a constant diffusion gradient of diffusing ions (diffusion agent) keeps up an unequal distribution of originally equally distributed ions (passive ions), the potential being related to the distribution of passive ions in the same logarithmic way as in the original Donnan equilibrium   It seems possible that in some similar way the continuous metabolism of the nerve cell is, by means of diffusing ionized metabolites, related to the nerve membrane potential

We know the reversible influence of inorganic ions on the potential To judge the probability of a relation as discussed above, it seems first of all necessary to know whether organic ions can exert a similar reversible influence, and if so, whether relations between such an

influence and the physical properties of the ions in question can be found

The present paper deals mainly with this question It shows a distinct, and to a certain degree reversible, influence of organic ions on the nerve injury potential, and, for the homologous series of the dialkylamines, a relation of the efficacy of an ion to its position in the series The interpretation of this relation will be discussed

Furthermore the results obtained on myelinated nerves are compared to those on non myelinated nerves, to test, in how far they are due to the specific nature of the myelin sheath and in how far to a nerve membrane, common to all types of nerves

Method

The nerves used were the sciatic nerve of *Rana pipiens* and the non myelinated limb nerve (mostly of the first and second leg, sometimes of the claw) of the spider crab, *Maia squinado* They were carefully prepared and usually immersed in oxygenated Ringer or sea water for about $1/2$ or 1 hour before the experiment

The active lead was made in Ringer or sea water, the inactive instead of using a cut, in isotonic KCl solution This yields a potential of 20–40 mv, which does not decrease due to recovery as in the case of a cut nerve. The slow fall of the potential which still takes place is due to a decrease of the potential at the active (Ringer or sea water) lead With good frog nerves it was sometimes only a few millivolts in 24 hours, with crab nerves more

A chamber of paraffin wax was used as shown in Fig 1 The nerve was drawn through four compartments, connected by hollow glass beads at the bottom Two were filled with purest mercury (redistilled, for use with electrodes) and served as seals (Control experiments with vaseline seals showed that the presence of mercury does not affect the potential during 24 hours) Of the other two which could be filled with solutions by means of an inlet and outlet, one contained isotonic KCl solution, the other Ringer (for frog nerves) or sea water (for crab nerves)

Leads were made through the inlets as shown in Fig 1C By means of agar bridges the calomel half cells could be connected successively with several chambers so that several nerves could be used simultaneously The calomel half cells were connected to a Leeds and Northrup student potentiometer and readings made with a box galvanometer (No 2420) of Leeds and Northrup, whose sensitivity was $1\ 3 \times 10^{-8}$ amp /mm scale, after focussing the image of the galvanometer out of the box on a scale at about 3/4 meter distance

To test the effect of a salt, the Ringers solution (or sea water) was replaced by an isotonic solution of the salt, mixed with Ringer (or sea water) in a ratio

A

K Cl   Hg   Sea water   Hg

Nerve

Inlet

Top view

B

K Cl   Hg   Sea water   Hg

Nerve

Outlet

Front view

C

KCl-agar   Ringer-agar   Nerve chamber

Calomel
half cell

KCl saturated
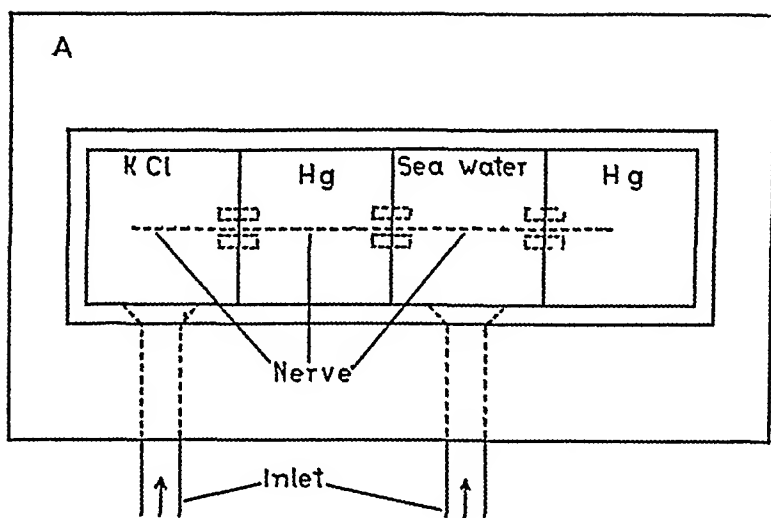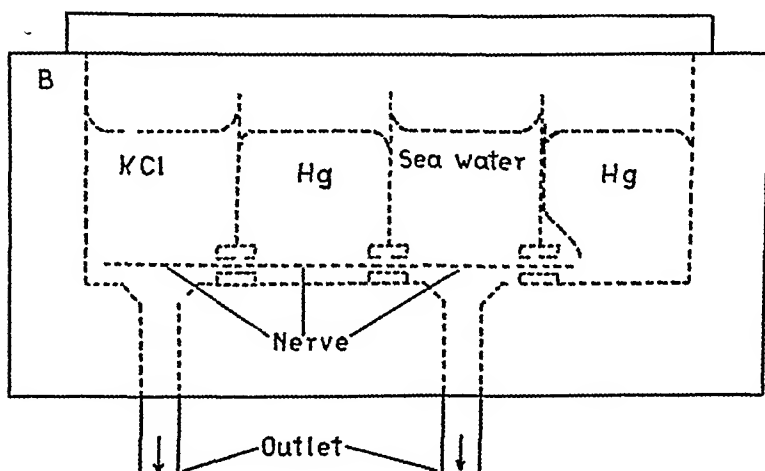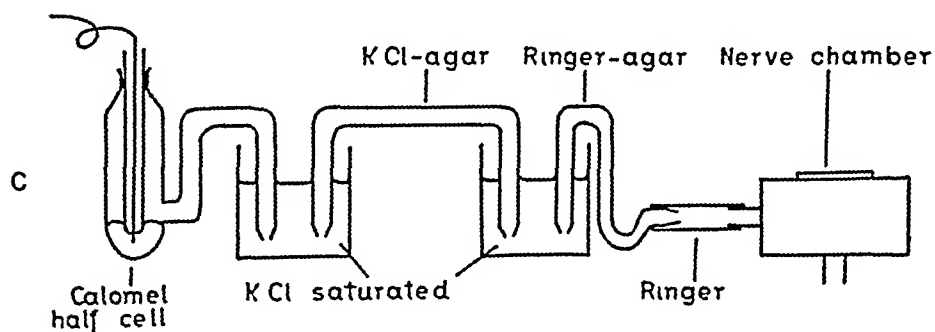
Ringer

Side view

Fɪɢ 1 Chamber of paraffin wax used in the experiments

varied according to the activity of the salt   1/10 KCl in the records means, for instance that Ringer was replaced by a mixture of 1/10 isotonic KCl and 9/10 Ringer   The salt solutions were neutralized to a pH of 7–7 5, when applied in Ringer, and about 8, when applied in sea water   The Ringer solution was buffered with phosphate   Fresh filtered sea water was used   After a certain time pure Ringer or sea water was introduced again to test the reversibility of the effect In the case of frog nerves, this time was about 1 hour, in the case of crab nerves about 10–15 minutes   Diffusion in frog nerves is very slow, due to the great amount of connective tissue around the nerve and between the fibers, whereas the crab nerves have little connective tissue and the nerves split into fine bundles as soon as they are immersed in sea water   Thus in the latter the nerve fibers are reached by the electrolyte much faster than in frog nerves   By splitting the connective tissue of frog nerves under the microscope with a quartz needle the diffusion time could be shortened considerably   The nerves seemed, however, to be damaged by the procedure and it was not used in the experiments

The salts used were the purest available preparations of Merck and Eastman Kodak   They were not further purified

Since the nerves showed considerable individual variations as to their response to ions several electrolytes were always tested on the same nerve and, especially between ions of about equal efficacy, comparisons were made only on the same nerve   Even then, however, the comparison was sometimes difficult due to the fact that the response of a nerve decreases with time

Effect of Inorganic Ions on the Potential of the Non Myelinated Nerve of the Spider Crab

Netter has investigated the effect of inorganic ions on the membrane potential of the sciatic nerve of the frog (Netter (1928))   He found that cations only affected the potential, the effect decreasing in the series $Li = Na < Cs < NH_4 < Rb < K$ Anions had no effect He concluded that the nerve membrane is solely cation permeable and consists of a pore system since the series he found was about that of the ionic sizes

As far as cations are concerned, the results in crab nerves agree fairly well with Netter's, as Fig 2 shows   Rb and K lower the potential in very high dilution, (1/40 was still found to be effective), Rb slightly more than K (Fig 2A)   NaCl in high concentrations (1/4 – 3/4 was mostly used) raises the potential a few millivolts (Fig 2B)   This is due to the dilution of the highly active K, as could be shown in experiments with artificial sea water, in which a corresponding lowering of the K concentration yielded the same result   Li
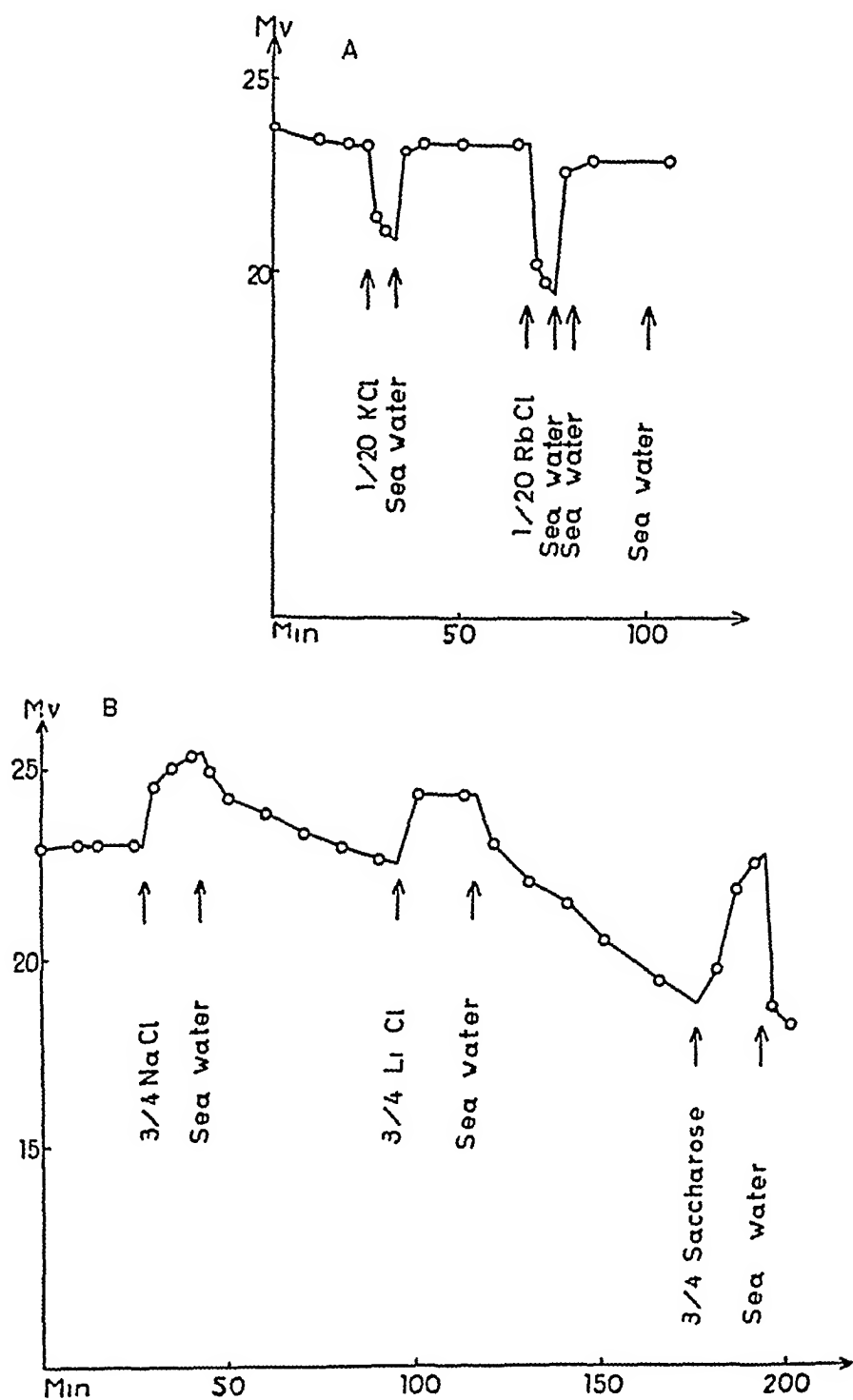
FIG 2 Effect of inorganic cations on the potential of crab nerves

has about the same effect as Na (Fig 2B) Both, however, although much less active than K and Rb, do also have a slight lowering in fluence on the potential This is shown when instead of NaCl or LiCl an isotonic solution of a non electrolyte (saccharose) equally diluted with sea water is applied Then, with equal dilution of the potassium, the potential rises considerably higher (Fig 2B), which shows the lowering effect of Na and Li
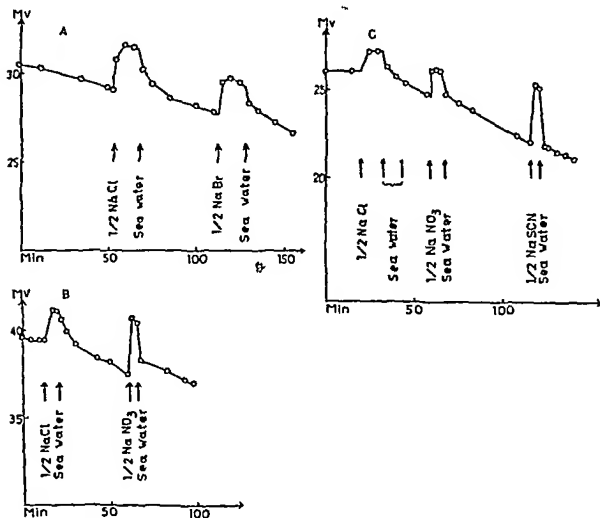
FIG 3 Effect of inorganic anions on the potential of crab nerves

The cations thus act in the series $Rb > K > Na = Li$, which agrees with Netter's results except the position of Rb

Anions, however, were found, in contrast to Netter's results, to have a distinct, if slight, effect on the potential Since they have the opposite sign their effect, if they are active, must of course be to raise the potential NaBr, NaNO₃, and NaSCN were used The dilution

of the potassium was taken into account by comparing the effect in each case with that of NaCl in the same concentration   Fig 3 shows, that, whereas NaCl and NaBr have about the same effect, $NaNO_3$ and, more definitely, NaSCN, raise the potential higher than NaCl does

Since the effect was only a few millivolts the possible interference of diffusion potentials was checked   A silk thread was used instead of the nerve in the same arrangement, and the effect of the salts tested in the same concentrations as on the nerve   The potential did not change by more than 3/10 mv   In the presence of 1/2 sea water diffusion potentials are depressed considerably

The series of activity of the anions is

$Cl = Br \leq NO_3 < SCN$ Their efficacy is about comparable to that of Li and Na, but much lower than that of K and Rb   The assumption of a completely anion impermeable membrane, therefore, cannot be made for the non-myelinated nerve   Whether it is strictly true for the myelinated nerve should be tested again   The very slow diffusion in the latter, together with the slightly injurious effect of SCN, might conceal a slight rise of the potential   It may be noted, that in Netter's paper evidence against the assumption of complete anion impermeability can be found   He found that the concentration effect of K was considerably higher than that of Na and Li, which is not possible for an exclusively cation permeable membrane for thermodynamic reasons   Accordingly collodion membranes with high concentration effect, i e nearly exclusive cation permeability, were shown to have the same concentration effect for all cations (Michaelis (1926))   The concentration effect on nerves is, of course, lowered by the intercellular short circuit, but this effect should be about the same for all cations

The Effect of Organic Ions on the Potential of Frog Nerves and Crab Nerves

A Cations —To avoid complications by hydrolysis only salts of strong bases were chosen dialkylamines, tetraalkylamines, guanidine, and choline

Fig 4 shows the effect of dialkylamines (from dimethylamine to diamylamine) on the potential of crab nerves   Under the influence of the lower members in high concentration, from dimethylamine to

dipropylamine, the potential changes very little, which means taking into account the dilution of potassium (cf Fig 2B), that their lowering effect is weak, but a little stronger than that of Na The effect decreases slightly from dimethylamine to dipropylamine From the next member on, dibutylamine, the tendency is reversed Dibutyl
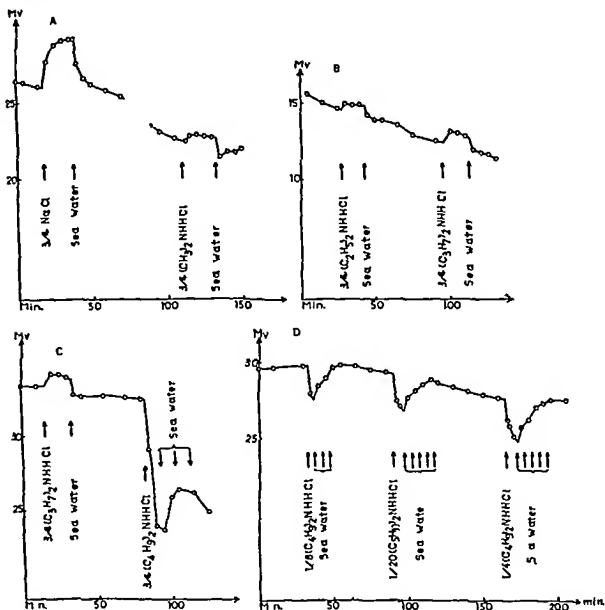
FIG 4 Effect of dialkylamines on the potential of crab nerves

amine has a strong effect, with the concentration 3/4 the potential falls in a few minutes by 10 mv but the fall is not completely reversible (Fig 4C) In a lower concentration, 1/8, a reversible effect can be obtained (Fig 4D) Diamylamine is still more active, even 1/20 lowers the potential rapidly and considerably, the efficacy being about

the same as that of K   In higher concentrations the reversibility is incomplete for this salt, too   This is a common feature of the active organic ions
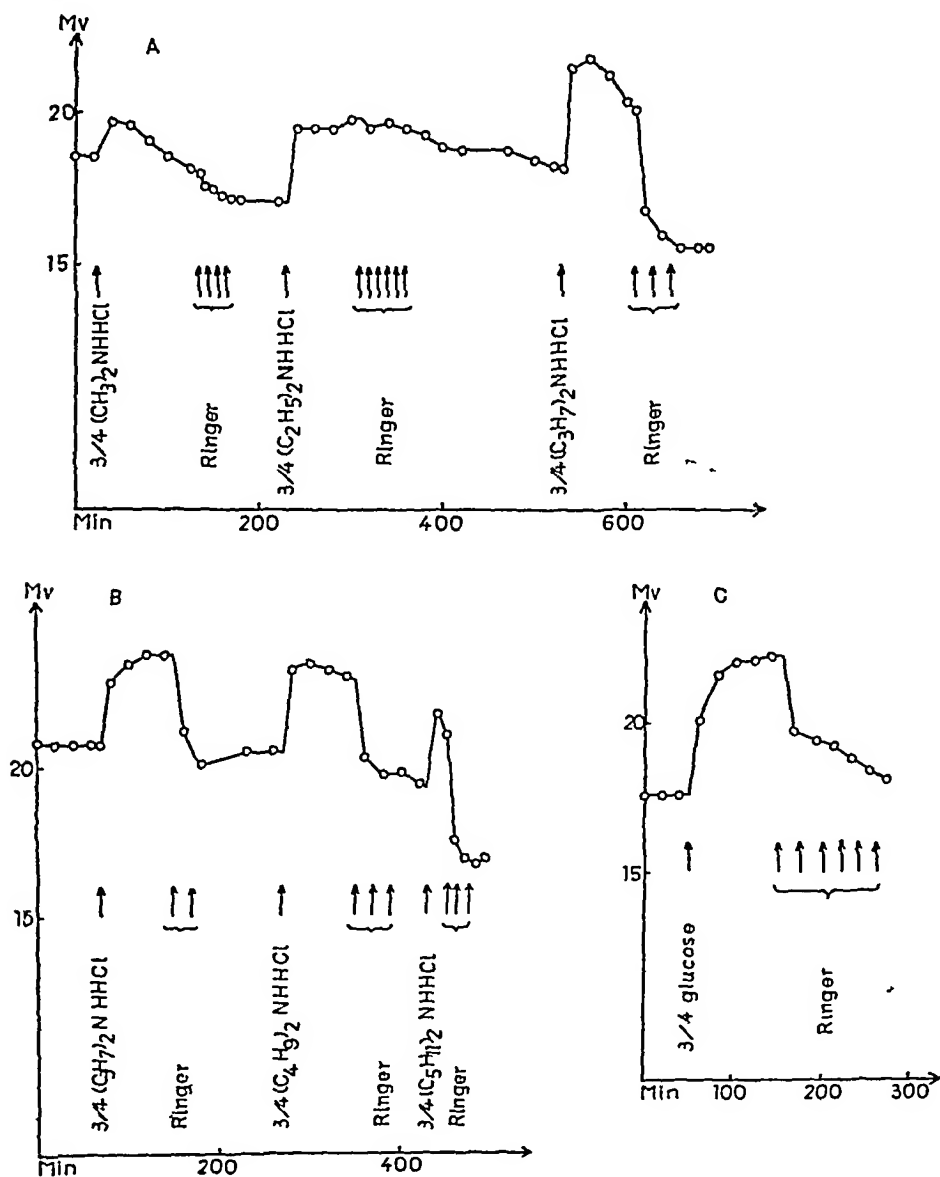
FIG 5 Effect of dialkylamines on the potential of the frog sciatic nerve

Thus, ascending in the homologous series, there appears a decrease first, and then an increase in the effect on the potential   The same is

true for the effect of the same salts on the potential of frog nerves, but the reversing point is different, dibutylamine is still inactive, only diamylamine shows a strong effect This is shown in Fig 5 Dipropylamine and dibutylamine are here nearly as inactive as a non electrolyte, glucose, dimethylamine and diethylamine a little more active, diamylamine very strongly active

The following interpretation of this striking behavior may be suggested In a porous system the ionic size determines the effect of an ion on the potential Thus, ascending in the series, a decrease of the effect would be expected in such a system This is what is true for the lower members Then, with increasing length of the hydrocarbon chain, another property prevails, the hydrophobic character of the ions This leads to their accumulation in the interface and thereby increases their activity, so to speak, by superposition of a concentration effect (Hober (1936))

This interpretation may seem arbitrary to those who like to regard the cell membrane as a homogeneous non aqueous layer and the objection may be made that instead of accumulation in the interface of a porous system, lipoid solubility may be assumed with equal justification, and the interpretation based on partition coefficients along the lines of either Osterhout's or Beutner's views The latter interpretation fails, however, to explain the decreasing effect of the lower members, for the hydrophobic character alone would lead to an increase of effect throughout the whole series The interfacial tension between an aqueous solution of dialkylamine and paraffin oil for instance, that may be taken as a measure of the hydrophobic character, decreases from the first member on This is shown in Table I, where the number of mm ^{3} per drop of dialkylamine solution formed from a stalagmometer in paraffin oil are listed The assumption, therefore, that partition coefficients determine the effect of the ions, seems not adequate to account for the results described, unless the assumption of a mosaic membrane is made to explain the behavior of the lower members Danielli (1935) has discussed the possible stability of such systems and found that their existence is not probable

An accidental observation made when measuring the interfacial tensions shown in Table I may be mentioned shortly If, instead of the non ionized interface water / paraffin oil, the ionized interface

water / olive oil + oleic acid is used, the interfacial tension increases
with increasing length of the hydrocarbon chain, instead of decreasing,
as Table II shows   The explanation seems to be, that the interface
active cations exert a discharging effect on the negatively charged
interface, and that the increase of interfacial tension due to this dis-
charge is greater than the decrease due to the molecular attraction

TABLE I

Interfacial Tension between Dialkylamine Solutions and Paraffin Oil
The salt solutions were N/50 in phosphate buffer N/72, pH 7 3

Salt	mm ³/drop
NaCl	234
Dimethylamine	231
Diethylamine	219
Dipropylamine	201
Dibutylamine	168
Diamylamine	151

TABLE II

Interfacial Tension between Dialkylamine Solutions and a Mixture of Olive Oil
and Oleic Acid 4 1
The salt solutions were N/50 in phosphate buffer N/72, pH 7 3

Salt	mm ³/drop
KCl	78 5
Dimethylamine	80 5
Diethylamine	80 5
Dipropylamine	99
Dibutylamine	114
Diamylamine	127

across the interface   Since biological interfaces are probably mostly
ionized, similar phenomena may have some biological importance
    If the interpretation given above is correct, it should be possible
to test it on a porous model   Michaelis has shown that the dried
collodion membrane is, in some respects, a useful model of the cell
membrane   That it is a porous system was shown in several ways
Non-electrolytes that do not disrupt the structure of the membrane,
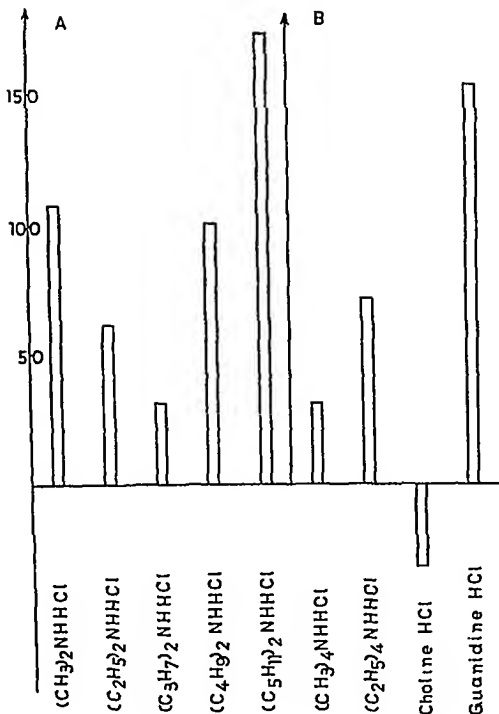
FIG 6 Potential differences across collodion membranes between organic salts N/100 and NaCl N/100 (in per cent of the potential difference across the same membrane between KCl N/100 and NaCl N/100) Sign positive on the side of NaCl in the external circuit.

penetrate through the dried membrane according to their molecular sizes (Michaelis and Weech (1928)), and the transition from incompletely dried membranes, that doubtless contain pores, to the com

pletely dried membranes is steady (Wilbrandt (1935))   The porous nature of this membrane may be regarded as certain, therefore

Fig 6 shows potential differences across dried collodion membranes between organic salts N/100 and sodium chloride N/100, in percentage of the potential difference measured between KCl N/100 and NaCl N/100   The dialkylamines show qualitatively the same behavior as on the nerve, in the lower part of the series the effect decreases with increasing length of the hydrocarbon chain, in the upper part from dipropylamine on, it increases (Fig 6A)

It should be noted that the results obtained on collodion membranes with organic ions, are not as reversible as those obtained with inorganic ions   Only fresh membranes were used, therefore, in which case the results were reasonably reproducible   On each membrane, to obviate individual differences, the potential difference between KCl and NaCl was first measured and then the potential difference between the organic salt and NaCl   (Therefore the values in Fig 6 are listed in percentage of the potential difference between KCl and NaCl)

Of tetraalkylamines only tetramethylamine and tetraethylamine could be tested   Tetramethylamine had no lowering effect even in the concentration 3/4   It raised the potential by dilution of the potassium, less than a non-electrolyte (Fig 7A), but more than dimethylamine (Fig 7B)   Thus, it is about as active as Na   Tetraethylamine is more active, it lowers the potential, (Fig 7C and 7D), thus being more active than the corresponding dialkylamine, diethylamine (in contrast to the methyl-compounds)   This is of some interest, because in his work on chemical stimulation J Loeb (Loeb and Ewald (1906)) also found tetraethylamine effective but tetramethylamine ineffective   In his interpretation of chemical stimulation he assumed a primary effect of the stimulus on the membrane potential, which is in accordance with our results   It is, however, striking that Netter found no effect of tetraethylamine on the potential of frog nerves On the other hand, our results on dialkylamines show that the effect of these ions is in general weaker on frog nerves than on crab nerves dipropylamine and dibutylamine, on frog nerves are about as inactive as a non-electrolyte (Fig 5B), whereas on crab nerves the first has a weak effect (but stronger than a non-electrolyte), the latter a rather
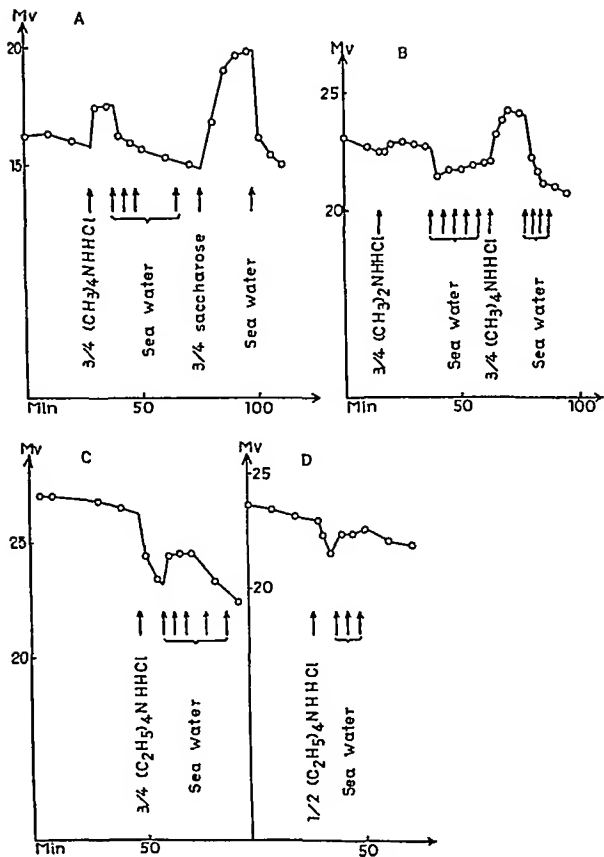
FIG 7 Effect of tetraalkylamines on the potential of crab nerves

strong effect (Fig 4C)   The reason for this difference will be dis-
cussed later

Guanidine has a strong effect, it lowers the potential even in the
concentration 1/8 (Fig 8A)   Choline is about as active as Na   It
raises the potential in the concentration 3/4 by dilution of potassium
(Fig 8B), but not as much as a non-electrolyte

Also the tetraalkylamines, guanidine and choline, act very similarly
on a collodion membrane, as Fig 6B shows tetraethylamine is stronger
than tetramethylamine, guanidine very strong, choline very weak
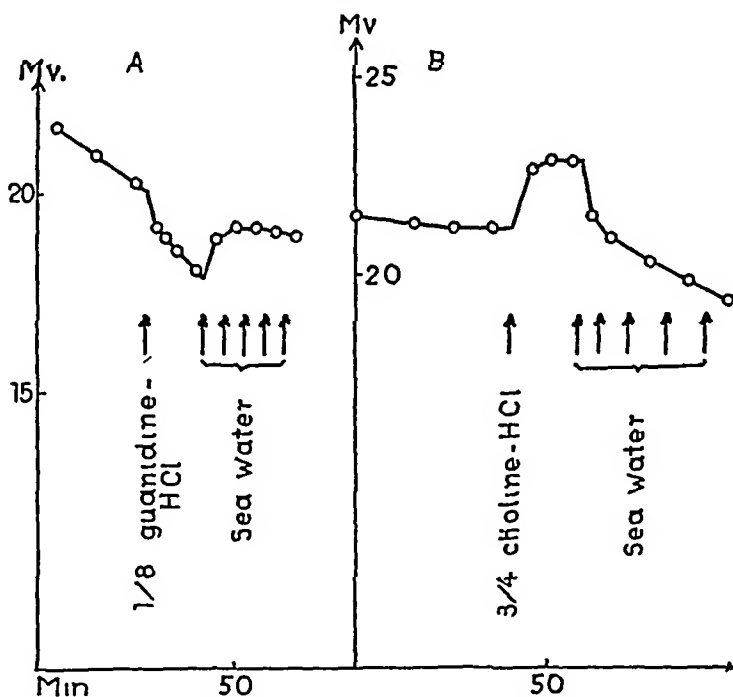
FIG 8 Effect of guanidine and choline on the potential of crab nerves

Summarizing the results with organic cations, we may state that an
influence of such ions on the nerve potential is definite   The effects
range between that of Na and K, according to the approximate series
Li = Na = choline = tetramethylamine < dipropylamine < dimeth-
ylamine = diethylamine < tetraethylamine < guanidine < dibutyl-
amine < diamylamine = K < Rb

The effects are, however, not as reversible as those of the inorganic
cations   Only after a very short application and in the lowest effective
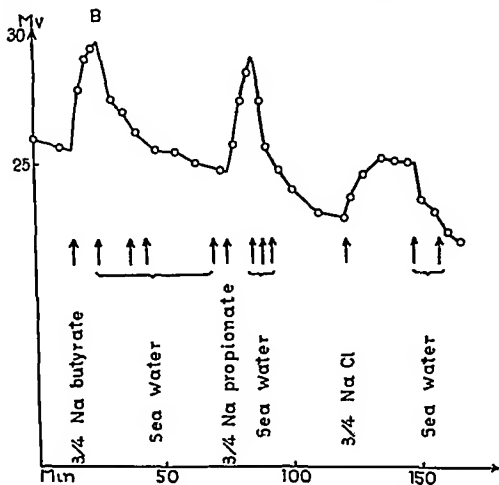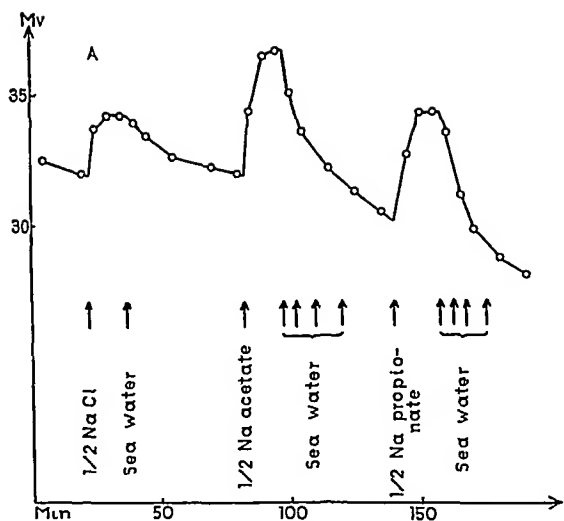
FIG 9 Effect of fatty acids on the potential of crab nerves

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concentrations is it possible to restore the original level of the potential
The effects resemble in a rather striking way those obtained on a
porous model, the dried collodion membrane

B Anions—Since an effect of inorganic anions on the potential
could be shown, and since the formation of acids during metabolism is
more frequent than that of bases, it appeared necessary for our prob-
lem also to test the influence of organic anions on the potential

Sodium salts of fatty acids from acetate to butyrate were used,
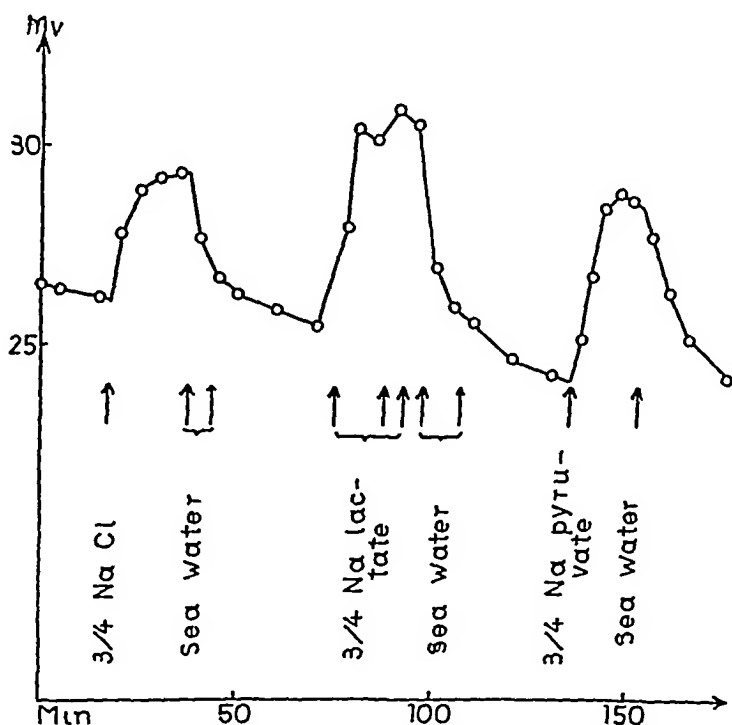
FIG 10 Effect of lactate and pyruvate on the potential of crab nerves

furthermore lactate and pyruvate   Fig 9 shows some records of the
effect of these salts   They exhibit a weak, but definite effect   In high
concentration they raise the potential more than is due to the dilution
of potassium as a comparison with the effect of NaCl in the same con-
centration shows   The effect, thus, is similar to that of the active
inorganic ions, NO₃ and SCN   A definite series among the fatty
acids could not be established, their action was about equal   Higher
acids could not be tested because they are too insoluble in sea water
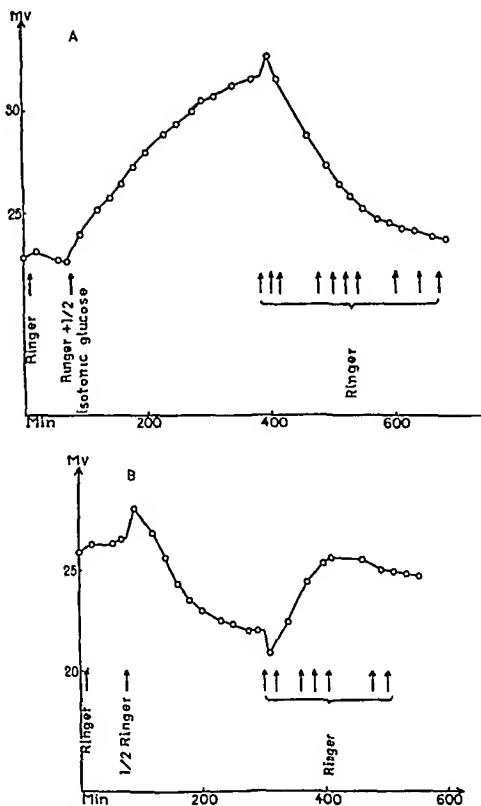
Fig 11 Effect of varied osmotic pressure on the potential of the frog sciatic nerve

Lactate and pyruvate were similarly active in high concentrations as Fig 10 shows

Summarizing we may state, that from the results also obtained with organic ions the assumption of a completely anion impermeable membrane around the nerve fiber cannot be supported   Organic anions have a weak, but definite effect on the potential

The Effect of Varied Osmotic Pressure on the Potential of Frog Nerves

Netter (1926–27) found that the frog sciatic nerve behaves like an osmometer, following the Boyle-Mariotte law   This finding seemed to furnish a possibility to test the fundamental assumption that the potential is a membrane potential due to the difference of ionic composition on the two sides of the membrane   If this is the case, osmotic compression of the nerve should raise the ion concentration inside, and, if it remains unaltered outside, i e if the surplus osmotic pressure outside is exerted by a non-electrolyte, the potential should rise   A decrease of the outside osmotic pressure brought about by dilution of the outside medium should, on the other hand, be followed by an expansion of the nerve and a dilution of the inside ions to the same extent that the outside solution has been diluted   Since the inside cations are mainly or exclusively potassium ions and the outside ions mainly sodium, and since the concentration effect of potassium on nerve has been shown by Netter (1928) to be greater than that of sodium, the potential should fall   Previous to the fall, however, before the water shift is completed, the potential should, of course, rise, due to a mere concentration effect   Fig 11 shows that both occur This seems to indicate that the assumption of a membrane potential is correct

It should be noted, that a similar effect on crab nerves could not be obtained   Since we know nothing about the osmotic properties of this nerve it is hard to interpret this negative result

<div align="center">DISCUSSION</div>

The results of this paper show a definite effect of organic ions, both cations and anions, on the membrane potential of the crab nerve   The efficacy of organic cations ranges between that of Na and K, that of anions is considerably weaker, but definite   The same is true for

inorganic anions   The membrane of crab nerves seems to be predominantly, but not exclusively, cation permeable similar to the findings of Hober for the membrane of muscles   The effects of organic ions are not as completely reversible as those of inorganic ions   If the ions are applied in the lowest effective concentration and only over a short time, however, the reversibility is complete

A relation of parts of the electrical disturbance to the metabolism of the nerve by means of ionized metabolites therefore seems possible

Whether the effect of ions formed inside the nerve fiber agrees with the effects described in this paper, where the ions were applied from outside, depends on whether the membrane is symmetric   This has mostly been tacitly assumed, for instance, when the effect of potassium from outside was used to interpret the resting potential as due to the high potassium concentration inside   The striking observations of Osterhout on the asymmetry of the membrane of *Valonia* and other large plant cells, however, have raised doubts   It should be pointed out, that in the latter case potential differences were measured across two cell membranes, whereas in the case of the nerve only one membrane is involved   The two cell membranes of *Valonia* are different and therefore the whole wall is asymmetric, but whether either of the two membranes is symmetric or not, we cannot judge from the experiments   Thus far there seems to be no evidence to indicate an asymmetry of a single cell membrane as far as the author knows

Assuming the membrane to be symmetric, we may briefly discuss the possibilities of a connection between electrical disturbance and metabolism   It is not probable that the spike is due to the formation of metabolites   Formation of active anions in very high concentration would have to be assumed to explain the direction of the change, but quantitatively their efficacy would have to be so considerably greater than those reported in this paper, that the assumption seems improbable   Formation of acids, however, might be related to the negative after potential, disappearance of acids or formation of bases to the positive after potential, especially since the after potentials seem to have a connection with the metabolism

The comparison of the non myelinated and the myelinated nerve showed large agreement, but definite differences in some details   The effect of organic cations, as far as it was studied, was in general stronger

on crab nerves   Particularly striking is the case of dipropylamine and dibutylamine, also tetraethylamine, which seems entirely ineffective on frog nerves   The same is true for the effect of inorganic anions It should be pointed out, however, that these differences are not necessarily due to differences of the cell membranes involved   It is entirely possible, that they are merely due to the fact that the salts are applied in one case in the presence of Ringer, in the other in the presence of sea water   The results reported here furnished some evidence that the hydrophobic character of the ions is important for their effect   This property is in general increased in a salt rich medium, for instance the solubilities of organic salts in sea water are mostly lower than in Ringer   It seems very likely, therefore, that the stronger effects of some organic ions on crab nerves in sea water are due to their increased hydrophobic character in this medium, in terms of our interpretation, to their increased interfacial accumulation

We may state that a difference between the membranes that are the seat of the potential of non-myelinated nerves and myelinated nerves could not be shown definitely   Non-myelinated nerves of land animals should be investigated to clear up this point

SUMMARY

1   The effect of osmotic pressure on the nerve resting potential of frog sciatic nerve is in accordance with the assumption of a membrane potential, increased osmotic pressure raises, decreased osmotic pressure lowers the potential

2   The potential of crab nerves is affected by organic and inorganic cations in the approximate series
$Rb > K$ = diamylamine > dibutylamine > guanidine > tetraethylamine > diethylamine = dimethylamine > dipropylamine > tetramethylamine = choline = Na = Li

3   The response of the potential to the series of dialkylamines (first decrease, then increase of response ascending in the series) is best understood by the assumption that the nerve membrane is a porous structure

4   With respect to these salts as well as to other organic cations the dried collodion membrane as a model of a porous membrane shows a striking parallelism to the nerve membrane

5 Both inorganic and organic anions (NO_3, SCN, acetate, propio nate, butyrate, lactate, pyruvate) have a definite, if slight, effect in raising the potential of crab nerves This effect of anions indicates that the nerve membrane is not completely anion impermeable

6 The effect of organic ions is, with certain restrictions, reversible Its possible relation to the resting potential and to the after potentials of the electrical disturbance is discussed

7 The response of the myelinated sciatic nerve of the frog and of the non myelinated nerve of the spider crab show considerable agreement There are some definite differences which are, however, not necessarily due to differences of the cell membranes involved, but may be ascribed to the difference of ionic conditions in Ringer and sea water

My sincere thanks are due to Prof R Hober, in whose laboratory this work was carried out, for the stimulation to the problem and for his continuous help and advice during the course of the work

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STUDIES IN BLOOD COAGULATION

V The Coagulation of Blood by Proteolytic Enzymes
(Trypsin, Papain)

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In 1916, Douglas and Colebrook noted that blood coagulation was accelerated by the addition of trypsin   A year later, Heard reported that trypsin could actually coagulate oxalated blood, and ascribed the phenomenon to a hypothetical disturbance in the Ca and P content of the fibrinogen, induced by the enzyme   These two reports were apparently overlooked by workers in the field   In 1928, Wald schmidt Leitz and his coworkers also reported that trypsin accelerated blood coagulation   They considered their experiments to prove that thrombin was a proteolytic enzyme either identical with or closely related to trypsin, that coagulation was due to the enzymic hydrolysis of fibrinogen to an insoluble modification, and that trypsin accelerated coagulation insofar as it hastened this hydrolysis   They found other proteolytic enzymes, including papain, to be inactive

Mellanby (1935 b) re investigated the action of trypsin on blood plasma and concluded that the coagulation observed by Heard was due simply to traces of calcium in the commercial preparations of trypsin, and was wholly unrelated to the enzyme as such

The present observations grew out of a chance re-discovery of Heard's observation certain mixtures of citrated horse plasma and trypsin were found to have clotted when examined after several hours   The participation of calcium could be excluded   Human, guinea pig, dog, horse, and rabbit plasma or blood could be similarly coagulated, provided only that one used the correct concentration of trypsin   Since the phenomenon promised to throw considerable light on the mechanism of physiological coagulation, it was studied in some detail

In addition to trypsin, papain was also found capable of initiating coagulation, but by a different mechanism   The latter is a true thrombin, insofar as it acts directly on fibrinogen to form an insoluble modification resembling fibrin   Trypsin, however, does not coagulate fibrinogen, but apparently reacts with plasma prothrombin to form the physiological coagulant thrombin

Methods and Materials

We are indebted to the Mulford Biological Laboratories at Glenolden for the citrated horse blood used in most of the experiments   The crude trypsin was the Digestive Ferments Company's "Trypsin 1 110," and the papain was a powder supplied by Merck   Both preparations were quite acid, and the suspensions in salt solution had to be cautiously neutralized before using   The suspended solids in such neutralized preparations were found to be relatively inert and could be removed by centrifugation

We are deeply indebted to Dr John H Northrop of The Rockefeller Institute for Medical Research at Princeton for a most generous supply of crystalline trypsin for use in these experiments   It is to be noted that the concentrations mentioned in the text are those of the dry powder, and this contains approximately 60 per cent $MgSO_4$

Preparation of Prothrombin —The precipitation of water-diluted plasma with acetic acid as described by Mellanby (1931) proved to be simple and to yield a more active product than the similar precipitation with CO_2 described by Eagle (1935a) in ignorance of the former procedure

Plasma was diluted with 15 volumes of cold (0-5°) water, and $N/1$ acetic acid was added until the precipitate became visibly granular   Usually, an amount of acid corresponding to 1/20 the original plasma volume sufficed

The centrifuged precipitate was dissolved in 0 85 per cent NaCl up to the original plasma volume, and neutralized cautiously with either $N/1$ $Na_2 CO_3$ or NaOH solution   The resultant opalescent solution contained fibrinogen, which could be removed by heating at 56° for 3–5 minutes, with little or no decrease in prothrombin activity of the solution (Eagle (1935a))

Alternatively, the prothrombin was purified by the Mellanby technic   The original acetic acid precipitate was suspended in water in a volume corresponding to 1/2 the original plasma volume, and this suspension then diluted with an equal volume of a 1/10 dilution of saturated Ca $(OH)_2$ previously brought to pH 7 0 with CO_2 After 10 minutes, the mixture was centrifuged and the supernatant fluid, containing a large proportion of the prothrombin and little or no fibrinogen, was rendered isotonic by the addition of 1/20 volume of 17 per cent NaCl

Preparation of Fibrinogen —Repeated precipitations (three to four) with 1 5 volumes of saturated NaCl yielded a satisfactory product which failed to coagulate on the addition of Ca and tissue extracts, but was promptly coagulated on the

addition of thrombin The final product was brought to a concentration of 0 9 per cent with respect to NaCl by proper dilution

Quantitative Estimation of Thrombin Activity —The time required for a given solution of thrombin to cause the coagulation of fibrinogen under standard conditions of temperature, volume, and salt concentration, was used as a criterion of its activity, and could be compared with the activity of other solutions by interpolation on a curve correlating coagulation time with thrombin concentration (Eagle (1935 a))

TABLE I

The Coagulation of Citrated Plasma by Trypsin

Coagulation time of 0 6 cc. plasma + varying quantities of trypsin solution in a total volume of 1 cc.

Plasma	Trypsin solution	Amount of trypsin solution *cc*							
		0 4	0 2	0 1	0 05	0 025	0 0125	0 0062	0 0031
	per cent			*min*	*min*	*m n*	*min*	*m n*	
Rabbit	10	∞ †	∞	1	$\frac{3}{4}$	1	5	35	
Guinea pig	10	∞	8 min	7	4	$5\frac{1}{2}$	—	—	
Human	10	∞	∞	8	5	3	$4\frac{1}{2}$	14	
Horse	7	∞	∞	$7\frac{1}{2}$	6	6	16	60	

* ' Difco 110 ' dissolved in salt solution   The results were unaffected by preliminary dialysis of the enzyme solution to remove possible calcium

† No clot in 24 hrs

The Coagulation of Blood or Plasma by Trypsin

A typical experiment which illustrates the fact that coagulation is observed only within a comparatively narrow optimum zone of trypsin concentration is given in Table I   That the clot results from the precipitation of the fibrinogen as a fibrillar gel resembling fibrin is clearly indicated by the following observations (1) plasma from which the fibrinogen is removed by heating for 5 minutes at 56°, or by half saturation with NaCl, can no longer be coagulated by the enzyme, (2) the supernatant fluid expressed from the clot contains no fibrinogen, as shown by heating to 56°, or by half saturation with NaCl, moreover, it no longer clots on the addition of either Ca or

thrombin in sufficient concentration to coagulate the original plasma, (3) the clot is microscopically indistinguishable from that which forms physiologically

The participation of calcium in the coagulation of plasma by trypsin was rendered improbable by the fact that coagulation was observed in trypsin plasma mixtures containing so much citrate and so little trypsin that even had the latter consisted solely of $CaCl_2$, no coagulation would have been observed Moreover, the coagulating activity of the trypsin was quantitatively unaffected by previous dialysis

Definitive proof that the observed coagulation was due to the trypsin *per se*, rather than adventitious substances such as Ca, was furnished by the fact that crystalline trypsin (Northrop and Kunitz) also caused coagulation of citrated blood or plasma, and was approximately 20 to 50 times as active in this respect as the crude trypsin It is true that the crystalline enzyme preparation contained $MgSO_4$. but the latter, in any concentration, failed to cause coagulation of citrated horse plasma, and if the trypsin was dialyzed against distilled water until it contained no demonstrable free SO_4, its coagulating activity was found to have been quantitatively unaffected

The Mechanism of the Trypsin Effect —Physiological coagulation involves two consecutive reactions In the first of these, a plasma factor (prothrombin) is activated by Ca and platelets (or tissue extracts) to form thrombin, subsequently, this thrombin reacts with fibrinogen to form fibrin It becomes of interest to ascertain how trypsin causes coagulation whether it *replaces one or more of these physiological reagents*, or whether the phenomenon is wholly unrelated to the physiological transformation of fibrinogen to fibrin

Trypsin was found to have no direct coagulative action on purified fibrinogen In sufficient excess, the enzyme digested the *protein*, and rendered it incoagulable even by thrombin but no clots were produced when trypsin in any concentration was mixed with fibrinogen (*cf* Table IV) Contrary to the thesis of Waldschmidt-Leitz and coworkers, trypsin is therefore not analogous to *thrombin*

Instead, the coagulating action of trypsin was found to rest on the fact that it reacts with prothrombin *to form thrombin, and this* thrombin then acts on fibrinogen to form fibrin If one adds varying

amounts of trypsin to a solution of prothrombin, and tests the coagu
lating properties of the mixture at intervals by adding aliquot por
tions to purified fibrinogen, one obtains results similar to those
illustrated in Fig 1 With too little trypsin, there is no formation
of thrombin There follows an intermediate range of concentrations,
in which a relatively slight increase in the amount of trypsin causes a
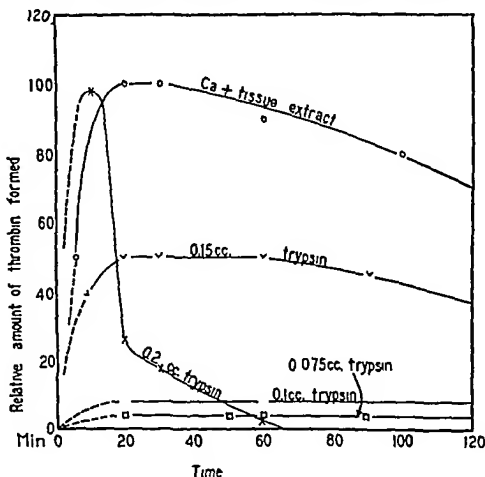
FIG 1 The formation of thrombin from prothrombin by trypsin Varying
amounts of 1/15 per cent crystalline trypsin were added to 2 cc of prothrombin
solution Aliquot portions were withdrawn at intervals and tested for thrombin
content A similar experiment was carried out, using $CaCl_2$ and tissue extract
instead of trypsin as the activating agent

disproportionate increase in the amount of thrombin formed At a
sharply defined optimum there is the rapid formation of a large
amount of thrombin This is usually between $\frac{1}{2}$ and $\frac{1}{5}$ the amount
formed from the same prothrombin by Ca and tissue extract, but in
some experiments the two thrombins are of approximately the same
activity With an excess of the enzyme, no thrombin formation can

Protocol 1

Thrombin is rapidly destroyed by the concentrations of trypsin necessary
for the activation of prothrombin (Fig 2)

To 1 6 cc of horse thrombin (prothrombin + Ca + lung extract) were added
0 4 cc of M/10 phosphate buffers and varying amounts of trypsin   The thrombin
activity of the mixtures was tested at intervals by adding aliquot portions to 0 4 cc
fibrinogen and noting the coagulation time   The curves given in Fig 2 represent
the amount of residual thrombin after 5 minutes   The results at pH 6 5, 6 2, 5 9,
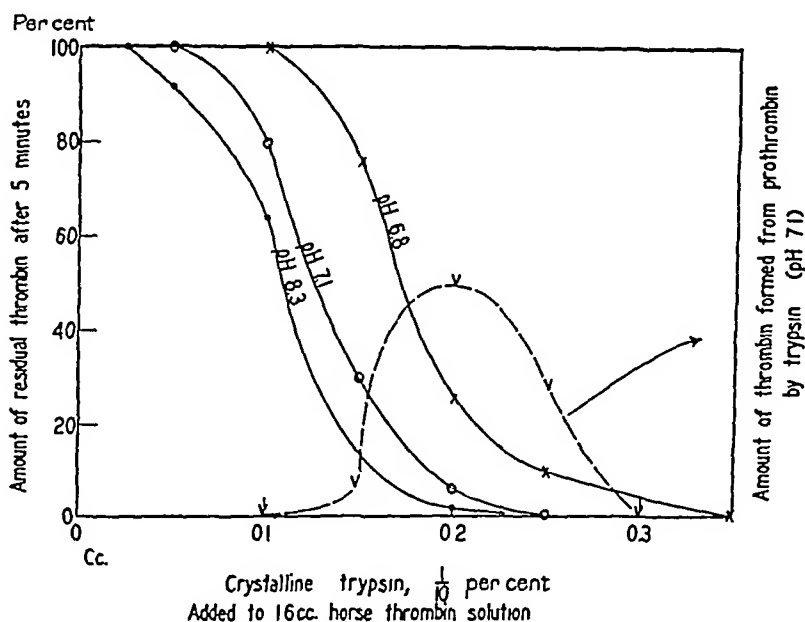
FIG 2   Thrombin is rapidly destroyed by the concentrations of trypsin neces-
sary for the activation of prothrombin

and 5 6 are not given, as they are practically identical with the curve at pII 6 8
The dotted curve in the figure represents the amount of thrombin formed from
the same prothrombin in 15 minutes by varying amounts of trypsin and it is
apparent that at the effective concentrations, thrombin is being destroyed almost
as fast as it is formed

In this experiment, as in that of Protocol 2, the pK' of the phosphate buffer
was arbitrarily taken as 6 8   The pH values given in the figures and tables are
therefore in error to the extent that the pK' of the several buffer-salt mixtures
deviates from 6 8 because of their varying ionic strength

be demonstrated, because of the digestion of prothrombin, thrombin, or both   It is to be noted that the thrombin formed at the optimum trypsin concentration is eventually similarly digested, and is often no longer demonstrable after 1 or 2 hours

The formation and destruction of thrombin proceed simultaneously (Protocol 1 and Fig 2), and the amount of thrombin demonstrated at any one time is a resultant of the two processes   As is shown in Fig 1, Table III, and Fig 2, this destruction is quite rapid at the
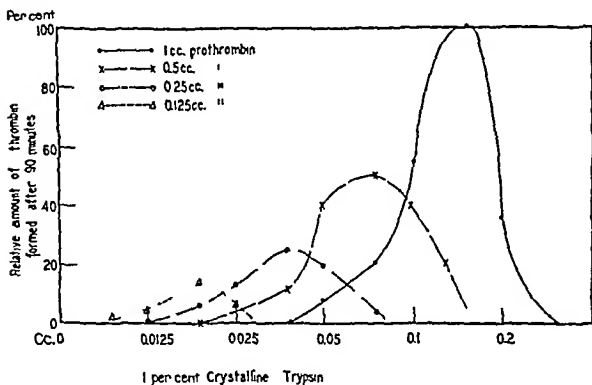
FIG 3 The reaction between prothrombin and trypsin to form thrombin Showing the linear correlation between the amount of prothrombin used, the optimum concentration of trypsin, and the amount of thrombin formed at the optimum trypsin/prothrombin ratio

optimum concentrations of trypsin, and explains the fact that the amount of thrombin elaborated by trypsin is usually only a fraction of that formed on the addition of Ca and tissue extract to the same prothrombin solution

The quantitative relationships between the amount of prothrombin solution used, the amount of thrombin formed, and the optimum concentration of trypsin are illustrated in Fig 3   As is there shown, there is a linear relationship between all three factors   The more

prothrombin used, the more thrombin is formed, and the greater is the amount of trypsin necessary to effect the transformation   Because of the progressive digestion of thrombin at the higher concen-

TABLE II

The Formation of Thrombin from Prothrombin by Trypsin Is Independent of the Presence or Absence of Tissue Extracts, Unlike the Physiological Transformation by Ca

To 2 cc of prothrombin prepared from Berkefeld-filtered horse plasma were added varying quantities of lung extract* and of crystalline trypsin, as indicated in the table   The numbers in the body of this table represent the relative amounts of thrombin formed   A control experiment using CaCl₂ instead of trypsin is also included

Tissue extract	Time of incubation	Relative amount of thrombin formed on addition of						CaCl₂, 0 2 cc of 0 1 м solution
		Crystalline trypsin, 1/15 per cent cc						
		0 3	0 2	0 15	0 1	0 075	0 05	
cc	*min*							
0 2		0	100	60	6	3	<2	125
0 04	10	0	100	50	6	3	<2	50
0 008		0	100	50	10	3	<2	10
0		0	100	50	8	3	<2	0
0 2		0	35	60	6	3	<2	90–100
0 04	20	0	25	60	6	3	<2	60
0 008		0	25	60	—	3	<2	15
0		0	25	60	6	3	<2	2–3
0 2		0	10	50	4	3	—	90
0 04	60	0	3	50	4	3	—	80
0 008		0	1–2	50	4	3	—	25
0		0	1–2	50	4	3	—	3

* An aqueous extract of rabbit lung tissue, previously washed free of blood, minced, and kept frozen until needed   Such extracts contained no demonstrable prothrombin or fibrinogen   The tissue extract could be replaced by a washed platelet suspension, which yielded the same results   a marked acceleration of thrombin formation in the presence of Ca, but no effect on thrombin formation by trypsin

trations of trypsin, the exact shape of the curves will vary according to the time interval allowed   Thus, if the experiment of Fig 3 were terminated after 15 minutes instead of 90 minutes, each of

the curves would be higher, and would be shifted to the right (less destruction of thrombin by the trypsin) but the relationships be tween prothrombin, thrombin, and trypsin there indicated would still hold

The optimum concentration of trypsin is determined largely by the protein content of the prothrombin solution The addition to the latter of inert serum, containing no demonstrable thrombin or prothrombin, causes a proportionate shift in the amount of trypsin necessary for thrombin formation The important factor is appar ently the amount of trypsin per unit protein, and the progressive shift in the optimum trypsin concentration as the amount of pro thrombin solution is increased (Fig 3) is an expression of this fact

The reaction between trypsin and prothrombin is independent of the presence of either Ca, platelets, or tissue extractives Pro thrombin prepared from Berkefeld filtered plasma, and which con tains neither Ca nor tissue extractive, is readily activated by trypsin, moreover, the rate of activation and the amount of thrombin formed are not affected by the addition of even large amounts of tissue extract (Table II)

An attempt to determine the optimum pH for the trypsin pro thrombin reaction is summarized in Protocol 2 and Table III As is there shown, the higher the concentration of trypsin, the more acid is the apparent pH optimum and this dependence on the trypsin concentration is accentuated if the trypsin prothrombin mixture is allowed to incubate for some time before the thrombin content is determined The discrepancy is explained by the data of Fig 2 Thrombin is rapidly destroyed only by the higher concentrations of trypsin, and this destruction proceeds more rapidly in the alkaline range than it does at pH 6 8 or 6 5, causing an acid shift in the ap parent optimum pH for thrombin production The experiment of Table III therefore offers no clue as to the true pH optimum for thrombin production by trypsin, but indicates instead how the amount of *net* free thrombin varies with pH, trypsin concentration, and time

That the coagulant produced by the interaction of trypsin and solutions of prothrombin is actually thrombin would seem to require no proof It is nevertheless of interest to note that if one adds trypsin

Protocol 2

The effect of pH on the trypsin-prothrombin reaction (Table III)

To 1 6 cc of horse prothrombin solution (heated at 56°C for 5 minutes to remove fibrinogen) were added 0 4 cc of м/10 phosphate buffers and 0 4 cc of varying concentrations of crystalline trypsin as indicated in Table III At the intervals there shown, aliquot portions of each solution were withdrawn and tested for thrombin activity by adding varying quantities to a fixed amount of similarly buffered fibrinogen and noting the coagulation time (*cf* page 545)

TABLE III

The Effect of pH on the Formation of Thrombin from Prothrombin by Trypsin

Crystalline trypsin, 1/15 per cent	Time interval	Relative amount* of thrombin formed at pH							
		8 3	7 4	7 1	6 8	6 5	6 2	5 9	5 6
cc	*min*								
0 4		0†	0	0	0	<3‡	<8	0	0
0 35		0	0	2 5	3	30	15	0	0
0 3	5	0	0	12 5	26	25	18	<8	0
0 25		1 5	8	12 5	35	20	17 5	<8	<10
0 2		9	13	10	12	10	8	<7	<10
0 175		4	3 5	3	3	1 5	2 0	0†	0
0 4		0	0	0	0	0	<5	0	0
0 35		0	0	0	0	26	27 5	0	0
0 3	15	0	0	7 5	30	45	50	40	<20
0 25		3	10	15 0	60	68	25	30	<20
0 2		12 5	18	17 5	16	17 5	12	6	—
0 175		3 5	3	2 5	2 5	2 0	2 0	0	0
0 4		0	0	0	0	0	0	0	0
0 35		0	0	0	0	0	0	0	0
0 3	90	0	0	0	1 5	3	10	15	<20
0 25		0	1 5	2 5	15	30	22	50	42
0 2		15	22 5	22 5	25	25	22 5	12	<20
0 175		—	—	—	—	—	—	3	0

* The amount formed from the same prothrombin by Ca and tissue *extract* is taken as 200

† Less than 1 on the arbitrary scale

‡ Soft clots or shreds form instead of a solid clot

In estimating the thrombin content of the solutions, it was necessary to take into consideration the fact that the coagulation time obtained with a given amount of thrombin varies with the pH Accordingly, a series of reference curves was constructed, correlating the coagulation time with thrombin concentration at the various pH levels used in the actual experiment, and the thrombin content of a given solution was obtained by interpolating the observed coagulation time on the appropriate reference curve.

in less than optimum concentrations to some prothrombin, and obtains, e g a 20 per cent yield of coagulant, and if one then adds Ca and tissue extractive, the total amount of coagulant formed is exactly the same as would have been formed by the Ca and tissue factor alone   Conversely, trypsin added to formed Ca thrombin (a pro thrombin Ca tissue extract mixture) has no effect other than to digest the coagulant if added in excess

Clearly, trypsin and the Ca tissue (or Ca platelet) system are mutually supplementary, and affect the same substrate, prothrombin, to form as end products coagulants which are qualitatively indis tinguishable   As has already been shown, the fact that the amount

Protocol 3

The coagulation of blood *in vivo* by trypsin

1   10 cc of a 5 per cent solution of crude trypsin were injected intravenously into a rabbit weighing approximately 2 kg   The animal died in convulsions in 2 minutes, and large clots were found in the great veins 2 minutes after death

2   1 cc. portions of a 5 per cent solution of crude trypsin were injected into a rabbit at 10 minute intervals   1½ minutes after the fourth injection it began to convulse, and was dead 1 minute later   On autopsy 6 minutes later the right ventricle and both auricles were found filled with a solid clot

3   10 cc of 5 per cent trypsin were injected intravenously into a rabbit   5 sec onds later, the animal began to convulse, and died within 2 minutes   The heart, opened 3½ minutes after death, contained a solid clot in every chamber except the left ventricle, which was fibrillating   Blood obtained from the great veins would not clot and no precipitate was obtained either on heating the plasma at 56 C for 5 minutes, or on the addition of 1 5 volumes of saturated NaCl

of thrombin formed from prothrombin at the optimum trypsin con centration is usually less than that elaborated by the addition of Ca is due to the rapid digestion of the formed thrombin by the trypsin

Prothrombin + Ca   ↗⟶

Prothrombin + platelets (tissue extractive) ↗⟶

Prothrombin + (Ca platelets)   ⟶   thrombin

Prothrombin + trypsin   ⟶   thrombin
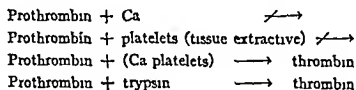
The implications of this analogy are discussed on page 557 Whether trypsin, as a proteolytic enzyme, hydrolyzes prothrombin to form thrombin, or whether trypsin combines with prothrombin to

form a new enzyme, thrombin, must be left unanswered for the present (*cf* page 557)

As is described in Protocol 3, trypsin injected intravenously into rabbits causes almost immediate death   Large blood clots are found in the heart and large vessels, the free fluid is non-coagulable, and contains no demonstrable fibrinogen   Trypsin thus seems to initiate blood coagulation *in vivo* as readily as it does *in vitro*

2 per cent papain solution
Amount added to 12cc horse prothrombin preparation
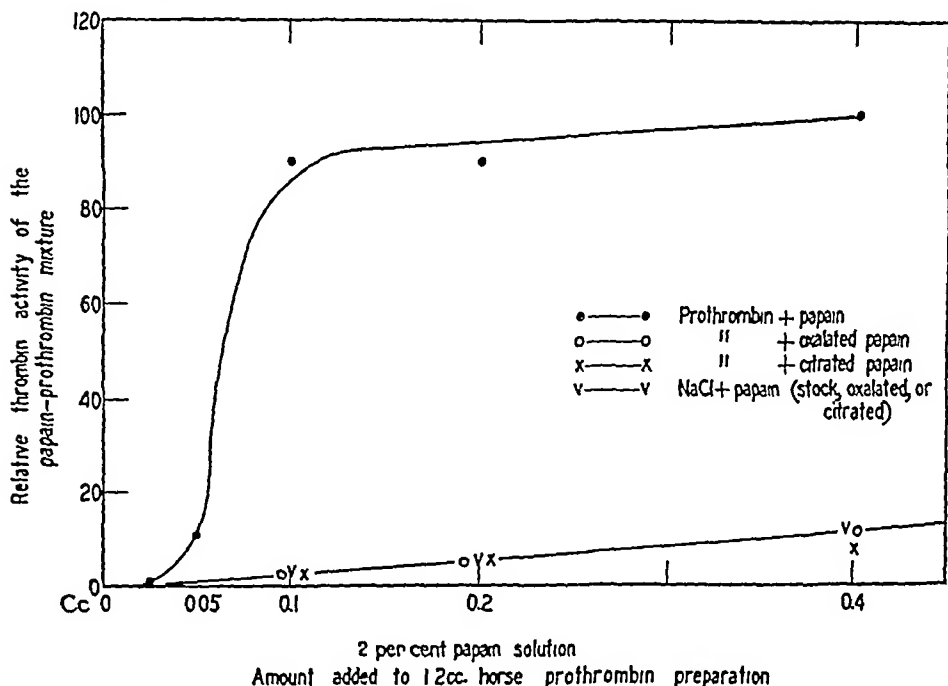
FIG 4 Papain converts prothrombin to thrombin only because of its Ca content but the enzyme as such coagulates fibrinogen   The prothrombin used in this experiment was the acetic acid precipitate heated at 56°C to remove fibrinogen (*cf* page 544), and contained sufficient platelet material to be activated to thrombin by the addition of Ca alone   The oxalated papain solution contained 0 016 M sodium oxalate, and was centrifuged free of the precipitated Ca oxalate   The citrated papain contained 0 15 M sodium citrate

The Coagulation of Blood or Plasma by Papain

In view of the results obtained with trypsin, it became of interest to ascertain whether other proteolytic enzymes had a similar effect Papain, the enzyme present in the juice of the papaw, was accordingly tested, and was found to be active   Unlike trypsin, however, papain

TABLE IV

Showing That Papain Unlike Trypsin Coagulates Fibrinogen 1s Such

Fibrinogen was precipitated from horse plasma by the addition of 1 5 volumes of saturated NaCl, the precipitate was redissolved in H₂O and reprecipitated for a total of 5 to 7 precipitations Aliquot portions of each successive solution were removed and tested for coagulability by trypsin papain Ca Ca + platelets, and Ca + platelets + prothrombin The figures in the body of the table indicate the coagulation time in minutes

No of precipitations of fibrinogen	Coagulability by trypsin						Coagulability by				Coagulability by papain				Interpretation
	Varying amounts of 0 1 per cent crystalline trypsin in a total volume of 0 4 cc. + 0 4 cc. fibrinogen solution						CaCl₂	CaCl₂ + platelets†	CaCl₂ + platelets + prothrombin‡	Formed thrombin§	0 1 cc. of fibrinogen + varying quantities of 5 per cent papain in a total vol of 0 8 cc				
	0 031	0 016	0 008	0 004	0 002	0 001					0 025	0 0125	0 0062	0 0031	
1	32	37	37	69	100		44	25	30	<2	2½	4½	9	22	Traces of residual prothrombin in the fibrinogen account for coagulability by trypsin and by CaCl₂ plus platelets
2		45	50	100	—			120	30	<2	3½	5	6	16	
3									40	<2	3	6	13	16	
4	No clot in 240 min						No clot in 240 min		60	<2	—	—	—	16	No residual prothrombin in fibrinogen no coagulation by trypsin, or by CaCl₂ + platelets The fibrinogen remains unchanged as regards its coagulability by papain or by thrombin
5									45	<2	6	8	11	15	
6									Not done	<2	5	5	15	12	

* 0 4 cc fibrinogen + 0 1 cc. CaCl₂, 1 per cent
† 0 4 cc. fibrinogen + 0 1 cc 1 per cent CaCl₂ + 0 1 cc. platelet suspension
‡ 0 4 cc fibrinogen + 0 1 cc CaCl₂ + 0 1 cc platelet suspension + 0 1 cc prothrombin
§ 0 4 cc fibrinogen + 0 1 cc thrombin solution

as such does not activate prothrombin to thrombin, but does act directly on fibrinogen to form a fibrillar gel resembling fibrin   As one repeatedly precipitates and redissolves fibrinogen, it becomes progressively less coagulable by either Ca + platelets or by trypsin, because of the rapid and eventually complete removal of prothrombin, but its coagulability by papain is unaffected (Table IV)   It should be pointed out that the crude enzyme powder contains large quantities of calcium, and accordingly activates prothrombin to thrombin   However, if the Ca is removed by the addition of oxalate or citrate, papain no longer activates prothrombin, but retains its coagulative action on fibrinogen quantitatively (Fig 4)

The clots formed by the action of papain are relatively soft, particularly when high concentrations of the enzyme are used, and in that range one observes a rapid re-solution of the clot analogous to the slow fibrinolysis occasionally observed after physiological coagulation

DISCUSSION

1   Crystalline trypsin has been found to activate prothrombin to thrombin   The available data offer no clue as to whether the prothrombin is merely hydrolyzed, or whether the enzyme actually combines with prothrombin to form a modified enzyme, thrombin   The minute amounts of trypsin which suffice are wholly compatible with a theory of stoichiometric combination   Thus, 0 003 cc of a 0 04 per cent solution of crystalline trypsin activated 1 cc of a prothrombin solution containing 0 04 per cent protein   The trypsin protein ratio was approximately 1 300, but since the actual prothrombin in a so called prothrombin solution is probably a very small fraction of the total solids present, the trypsin prothrombin ratio in this experiment may well have been 1 50, 1 5, or even 1 1

2   The terms cytozyme, thrombozyme, and thrombokinase which have been applied to the platelet (or tissue) factor by various investigators express their belief that this factor functions as an enzyme in the activation of prothrombin to thrombin   The only objective evidence hitherto presented in support of this hypothesis is (a) the minute amount of platelets or tissue extractive which suffices to produce a maximum yield of thrombin, and (b) the fact that further

increase in the amount of platelets merely accelerates thrombin production and does not affect the quantity formed (Eagle (1935 a))

The present experiments show that trypsin has the same activating effect on prothrombin as the physiological system, Ca + platelets (or Ca + tissue extracts) It is difficult to conceive of so specific a phenomenon as the transformation of prothrombin to thrombin being effected by two wholly dissimilar mechanisms We therefore suggest as a tentative working hypothesis that Ca + platelets together constitute a proteolytic enzyme analogous to trypsin,[1] which reacts with prothrombin to form thrombin As in the case of trypsin, there is no experimental evidence which justifies a choice between the theory that the platelets combine with prothrombin to form thrombin, and the alternative possibility that the transformation merely involves enzymic hydrolysis

We have been unable to demonstrate any proteolysis (formol titration) during the Ca platelet prothrombin reaction but it is entirely possible that such proteolysis occurs and escapes detection because of the minute molecular concentration of actual prothrombin in the preparations used

3 The effect of trypsin here described has several other important implications Howell and Fuchs maintain that prothrombin is present in circulating blood as an inactive heparin prothrombin compound, that platelets combine with heparin and thus release free prothrombin, and that this free prothrombin then reacts with Ca to form thrombin [2] The present experiments clearly show that Ca is not necessarily an intrinsic part of thrombin, for trypsin reacts directly with prothrombin to form thrombin in its complete absence Again, according to the theory of Howell and Fuchs, platelets or tissue extracts mobilize prothrombin from its hypothetical union with heparin, and should therefore increase the rate and degree of thrombin production, whether one uses Ca or trypsin as the activating agent As is illustrated in Table II, however, tissue extracts have no demonstrable effect on the rate or degree of thrombin formation by

[1] The postulated platelet enzyme is clearly not trypsin as such for trypsin rapidly digests formed thrombin, while Ca and platelets have little or no effect

[2] According to Fuchs, platelets function both in the hypothetical mobilization of prothrombin, and in the subsequent reaction with calcium

trypsin, and similar results have been obtained on using platelets instead of tissue extracts   It would therefore seem that the latter reagents do not function physiologically by freeing prothrombin from a hypothetical union with heparin   It is significant in this connection that trypsin injected intravenously coagulates circulating blood as readily as it coagulates plasma *in vitro*, the prothrombin of circulating blood is apparently free and reactive   There are other reasons to doubt that heparin is of physiologic significance in preventing intravascular coagulation (Quick, Mellanby (1935 *a*), Eagle, in preparation), and taken in conjunction with the experiments cited, they render the heparin theory improbable

By the same token, the fact that trypsin can cause intravascular coagulation, seems to invalidate the theory of Bordet that circulating blood contains, not prothrombin as such, but an inactive precursor which becomes reactive only on contact with some foreign surface, such as glass

4 A second proteolytic enzyme, papain, was found to have no effect on prothrombin other than its digestion, but was found to act directly on fibrinogen to form a fibrillar gel resembling fibrin   If one considers this product to bear only a superficial resemblance to fibrin, then the phenomenon is of no significance as regards physiological coagulation, but if the clot formed by papain is identified as fibrin, it becomes probable that thrombin, like papain, is a proteolytic enzyme

This was the theory originally proposed by Schmidt in 1872, and the present experiments indicate that it is still the most satisfactory working hypothesis as to the mechanism of thrombin action (*cf* Eagle (1935 *b*))

5 Experiments now in progress indicate that the coagulating activity of proteolytic enzymes offers an adequate and simple explanation for the well known but hitherto unexplained observations that certain bacteria and certain snake venoms can cause plasma to coagulate and they suggest a new attack (leucocytic enzymes) on the mechanism of fibrin deposition at sites of inflammation

6 It is to be noted that the recently discovered activation of chymo-trypsinogen by trypsin, with the *formation of chymo-trypsin*

(Kunitz and Northrop), offers a complete analogy for the mechanism of physiological coagulation as here suggested

Substrate		Activating proteolytic enzyme		Product, a proteolytic enzyme
Chymo trypsinogen	+	Trypsin	$\longrightarrow$	Chymo-trypsin
Prothrombin	+	Trypsin Ca platelets Ca tissue extracts Snake venoms* Bacterial proteases*	$\longrightarrow$	Thrombin a proteolytic enzyme analogous to papain and certain snake venoms* in that it converts fibrinogen to fibrin

* To be discussed in following papers

SUMMARY

Crude or crystalline trypsin in proper concentration causes the blood or plasma of human beings, dogs, rabbits, guinea pigs, and horses to coagulate It does not clot the fibrinogen directly, but reacts with prothrombin to form thrombin Since trypsin thus has the same effect as the physiological system Ca plus platelets (or Ca plus tissue extracts), it is suggested as a tentative working hypothesis that the latter system contains a proteolytic enzyme with a specific affinity for prothrombin Other implications of this trypsin effect with respect to the mechanism of physiological coagulation are discussed in the text (pages 557–558)

The proteolytic enzyme papain also coagulates blood In this case the enzyme does not activate prothrombin, but acts directly on fibrinogen to form a fibrillar gel resembling fibrin If one admits this clot to be fibrin, this constitutes strong evidence that thrombin, the physiological coagulant, is also a proteolytic enzyme with a specific action on fibrinogen

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THE ESTIMATION OF PAPAIN WITH HEMOGLOBIN

BY M L ANSON

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Papain, like trypsin, digests proteins such as hemoglobin and casein most rapidly in slightly alkaline solution   The hemoglobin method used to estimate trypsin (Anson and Mirsky (1933–34)) can be used without change to estimate papain, provided papain is properly activated with cyanide and there is sufficient cyanide in the hemo globin solution   Denatured hemoglobin at pH 7 4 is digested for 5 minutes at 25°C and the split products not precipitated by trichlor acetic acid are estimated colorimetrically with the phenol reagent which gives a blue color with tyrosine, tryptophane, and cysteine By this method only the first stage of digestion, due to proteinase, is measured, for hemoglobin needs to be only slightly digested for the digestion products to be not precipitable by trichloracetic acid

The digestion of hemoglobin by papain, as measured by the tri chloracetic acid method, is increased by cyanide and cysteine and abolished by iodoacetate, even in the presence of cyanide   Thus the effect of iodoacetate on the digestion of protein by commercial papain, unlike the effect of iodoacetic acid on digestion by liver extract (Anson (1936–37)) is an effect on the proteinase   In the procedure to be described papain is activated by cyanide in strongly alkaline solution In neutral or acid solutions, such as have hitherto been used, activa tion is less rapid and complete

Activation—To 0 5 ml enzyme solution are added 5 drops of 2 N sodium cyanide and after the solution has stood 3 minutes at 25 C 9 25 ml water   1 ml of this solution is usually used for estimation   If the solution is diluted further the dilution is carried out with a solution containing 5 drops of 2 N sodium cyanide in 10 ml   Increasing the time papain is incubated with sodium cyanide to 10 minutes, increasing the amount of sodium cyanide to 10 drops, or raising the temperature to 37°C does not change the activity of the papain more than 3 per

cent   If cyanide is not added the activity is negligible   If 5 drops of 1 8 M potassium acid phosphate are added in addition to 5 drops 2 N sodium cyanide the activity is 40 per cent of the activity obtained with the cyanide alone   I have not studied the activation of purified or modified papain

If 10 gm of commercial papain is stirred with 100 ml of water and the suspension is filtered, the filtrate is diluted 20–25 times before the 5 drops of sodium cyanide are added   That is, the extract of about 0 2 mg papain is used for estimation   If the 20–25 times dilution is carried out with water, the 5 drops of 2 N sodium cyanide are added immediately since the enzyme slowly becomes destroyed
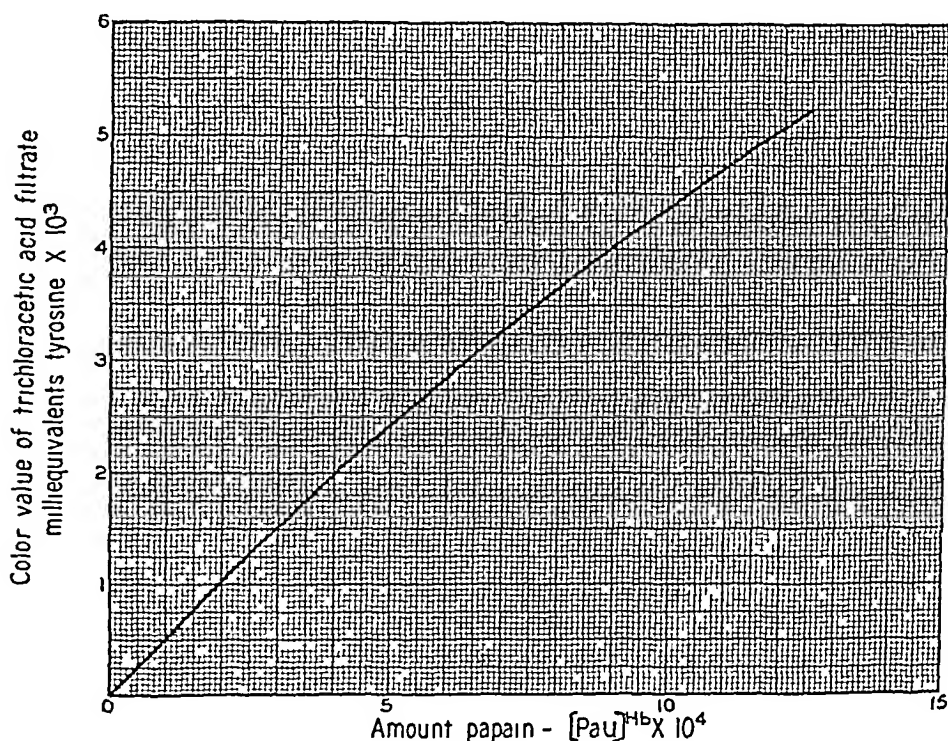
FIG 1   Relation of amount of papain used for 5 minutes digestion of hemoglobin at 25°C and color value of trichloracetic acid filtrate

in dilute solution   If the dilution is carried out with dilute cyanide, the enzyme is stable

Estimation —The preparation of the hemoglobin solution, the carrying out of the estimation, and the calculation of the results, are exactly the same as in the estimation of trypsin by hemoglobin (Anson and Mirsky (1933–34))   Digestion is for 5 minutes at 25°C   The extent of digestion produced by a given amount of papain in 5 minutes is the same within 3 per cent as that by one-fourth the amount of papain in 20 minutes   Fig 1 gives the curve relating the extent of digestion to the amount of enzyme used

Definition of Activity Unit $[Pa\ u]^{Hb}$ —The symbol $[P\ u]^{Hb}$ is not used because it stands for pepsin unit (Anson and Mirsky (1932–33)) The initial rate of digestion at 25°C by 1 ml of papain solution containing 1 $[Pa\ u]^{Hb}$ of papain is such that there is produced per minute in 6 ml of digestion mixture an amount of color producing substance not precipitable with trichloracetic acid which gives the same color with the phenol reagent as 1 milliequivalent of tyrosine

Influence of Various Factors —To test the influence of the various factors on the extent of digestion, after activation with 5 drops of 2 N sodium cyanide, the papain solution is diluted to 5 ml instead of 10 ml and 0 5 ml is used for digestion instead of 1 ml   The addition of 0 5 ml of the following solutions has, within 3 per cent, the same effect on the extent of digestion in 5 minutes as the addition of 0 5 ml water 0 2 N sodium hydroxide, 0 1 N hydrochloric acid, 9 M urea, 0 1 M ammonium sulfate, and glycerine   The addition of 0 5 ml 0 02 M iodoacetic acid makes the digestion negligible   The extent of digestion as measured in the standard way is the same whether 5 ml hemoglobin solution is used or 4 ml hemoglobin solution plus 1 ml water

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THE ESTIMATION OF CATHEPSIN WITH HEMOGLOBIN AND THE PARTIAL PURIFICATION OF CATHEPSIN

BY M. L. ANSON

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An account of previous work on cathepsin may be found in the monograph of Pozzi (1935)

In the hemoglobin method for the estimation of proteinase, denatured hemoglobin is digested and the digestion products not precipitable by trichloracetic acid are estimated colorimetrically Hemoglobin, unlike commercial casein, edestin, and gelatin, is a reproducible substrate Only the very first stages of digestion are measured by the trichloracetic acid method, for only a small amount of digestion is needed to make hemoglobin not precipitable by trichloracetic acid Thus, only true proteinase is estimated by the hemoglobin method

Almost all the estimations of proteinase recorded in the literature have been made with substrates that are not reproducible As a result, the values of proteinase activity obtained in different laboratories, or in the same laboratory with different batches of substrate, are not quantitatively comparable Furthermore, most of the values of proteinase activity recorded, in particular those obtained by the Willstatter school, were obtained by methods which estimate not proteinase activity alone but the activity of proteinase plus other proteolytic enzymes As a result, the values obtained by different investigators are not even qualitatively comparable The hemoglobin trichloracetic acid method estimates proteinase alone, yields reproducible results, and has now been applied to the four known types of proteinase, pepsin (Anson and Mirsky (1932–33)), trypsin (Anson and Mirsky (1933–34)), papain (Anson (1936–37)), and cathepsin It would seem desirable that if some other method is used, either independent evidence be given that the method estimates proteinase

alone and yields reproducible results, or that the results obtained by the other method be compared with those given by the hemoglobin method

The hemoglobin solution used for the estimation of cathepsin (2 per cent denatured hemoglobin in 0 2 N acetic acid) has a pH of 3 7   The rate of digestion can be decreased but not increased by the addition of acid or alkali   Pepsin has at pH 3 7 only a small portion of its activity at pH 2 5   At pH 2 5 cathepsin is rapidly destroyed

The activity of liver cathepsin, as measured by the hemoglobin-trichloracetic acid method, is not affected by the addition to 6 ml of digestion mixture of 1 ml of 0 1 M cysteine, 0 02 M copper sulfate or 0 01 M iodoacetic acid   Cathepsin is not a proteinase of the papain type   There is no reason to believe that proteinase action in living cells or the first stages of proteolytic autolysis is regulated by heavy metal and sulfhydryl compounds

If the hemoglobin solution used for the estimation of cathepsin is too acid, cathepsin is destroyed in the hemoglobin solution   Cysteine slows but does not stop this destruction   In an acid solution in which cathepsin is unstable, therefore, cysteine increases the rate of digestion This cysteine effect is neither activation of the enzyme nor removal of an inhibitor

In the standard hemoglobin solution cathepsin is stable   Although to estimate cathepsin digestion has, in the past, usually been carried out for 24 hours at 37°C , in general the stability of cathepsin in the substrate solutions used has not been tested

Liver extract contains, in addition to the proteinase (or group of proteinases) called cathepsin, other proteolytic enzymes which digest further the split products produced by cathepsin   The rate of this further digestion is increased by cysteine and decreased by iodoacetic acid   The incorrect conclusion that cathepsin is a proteinase of the papain type was based on supposed estimations of proteinase which were in reality estimations of cathepsin plus other proteolytic enzymes

The digestion of globin by liver extract can be followed either by the trichloracetic acid method or by formol titration   As measured by the trichloracetic acid method, which estimates only proteinase, the rate of digestion of globin, like that of hemoglobin and casein, is not influenced by cysteine or iodoacetic acid   As measured by the formol

titration method, which estimates the total action of all the proteolytic enzymes, the rate of digestion of globin in the same solution is increased by cysteine and decreased by iodoacetic acid, even when the increase in formol titration for 6 ml of digestion mixture is only 1 ml of 0 02 N sodium hydroxide

In the presence of iodoacetic acid, liver extract produces no detectable increase in the formol titration of a cysteine containing gelatin solution of pH 3 2 Gelatin, the most commonly used substrate for the estimation of cathepsin, is digested by cathepsin only slightly, if at all It is generally assumed that iodoacetic acid inhibits enzyme action by combining with SH groups The combination of iodoacetic acid with SH groups at pH 3 2, however, has not yet been demonstrated

In the estimation of cathepsin by the hemoglobin method not only is the digestion solely due to proteinase, but only the first stages of digestion by proteinase are measured, for two reasons First, the greater the extent of digestion, the less is the percentage increase in the extent of digestion due to a given increase in the amount of enzyme used or in the time of digestion Secondly, the greater the extent of digestion, the greater the inhibition by split products and the greater the possibility of other proteolytic enzymes influencing the rate of digestion by destroying inhibitory split products

Before the hemoglobin method is used for the estimation of any proteinase of the cathepsin type, the optimum pH, the shape of the digestion curve, and the effect of iodoacetic acid should first be determined For instance, the cathepsin of bakers' yeast digests hemoglobin most rapidly at a pH more alkaline than that at which liver cathepsin digests hemoglobin most rapidly For the estimation of yeast cathepsin, 0 6 ml of 1 N sodium hydroxide is added to each 10 ml of the standard hemoglobin solution used for the estimation of liver cathepsin The digestion by yeast cathepsin, like that by liver cathepsin, is not affected by iodoacetic acid

Preparation of Hemoglobin —Whipped beef blood is centrifuged, the serum and white blood corpuscles are siphoned off, the red corpuscles are stirred with an equal volume of cold 1 per cent sodium chloride solution centrifuged again and stored frozen The corpuscles are dialyzed overnight against cold distilled water in cellophane tubing in a shaking dialyzer to remove dialyzable color producing

substances   On one occasion hemoglobin dialyzed against running tap water in a large jar gave satisfactory results   The concentration of the dialyzed hemoglobin is estimated by the color it gives with the phenol reagent   Water is added to give a solution containing 10 gm hemoglobin in 100 ml of solution, and the solution is stored at 5°C with toluol as a preservative   1 5 mg of hemoglobin gives the same color as 0 15 mg of tyrosine

Preparation of Substrate Solution —First 40 ml of 5 N acetic acid and then 200 ml of 10 per cent dialyzed hemoglobin are added to 760 ml of distilled water previously heated to boiling   The solution is cooled with tap water, shaken with toluol, allowed to come to 5°C in a refrigerator, filtered with suction with the aid of Standard Super-Cel (Johns-Manville) and the filtrate is stored at 5°C with toluol as a preservative

The experiments described in this paper were carried out with hemoglobin substrate prepared as just described   The substrate solution, like the other hemoglobin substrate solutions, contained a little toluol as a preservative   Recently Dr M Kunitz found that a *large* amount of toluol decreases the rate of digestion of the hemoglobin substrate by trypsin   There is a similar but smaller effect of toluol on the digestion of hemoglobin by pepsin and cathepsin   The effect on cathepsin can be diminished but not completely abolished by a preliminary removal of the stromata with ether and sodium chloride   As a result of these observations toluol is no longer used at any stage of the preparation of any of the hemoglobin substrates   The washed red blood corpuscles are dialyzed as soon as they are prepared and then frozen   In the preparation of the cathepsin substrate after the hemoglobin is added to the hot acetic acid solution, the solution is promptly cooled and then can be used immediately   1 mg of the preservative, merthiolate (Lilly), is added to each 50 ml solution   According to the manufacturer, this small amount of preservative suffices to prevent bacterial growth   Larger amounts of merthiolate give an appreciable color with the phenol reagent

For the estimation of purified spleen cathepsin with hemoglobin I now use 2 per cent hemoglobin dissolved in 0 4 N acetic acid   The use of the stronger acid does not change the rate of digestion but it makes the rate of digestion less sensitive to the addition of alkali

Estimation —1 ml of enzyme solution is added to 5 ml of hemoglobin solution Digestion is carried out at 37°C for a length of time depending on the amount of enzyme used, but never for less than 10 minutes   Digestion is stopped by the addition of 10 ml of 0 3 M trichloracetic acid   (Trichloracetic acid should be titrated rather than weighed, for commercial samples of trichloracetic acid contain varying amounts of water)   The suspension is filtered   To 5 ml of filtrate is added rapidly 10 ml of 0 5 N sodium hydroxide and in fast drops, with shaking, 3 ml of the phenol reagent of Folin and Ciocalteau (1927) diluted with twice its volume of water   The resulting blue solution is estimated colorimetrically after 1–5 minutes in the fairly monochromatic light transmitted by the Corning glass

filter No 241, with a blue glass as a standard The blue glass is calibrated with the blue solution obtained by the reaction of the phenol reagent with 0 15 mg tyrosine For the color development with tyrosine 5 minutes are allowed

If copper sulfate is present in the digestion mixture, then 1 5 ml of trichloracetic acid filtrate is used for estimation To this 1 5 ml are added 4 9 ml of water, 1 drop of 0 1 M copper sulfate, and 8 6 ml of 0 5 N sodium hydroxide The smaller amount of filtrate is used because copper sulfate increases the color given by the phenol reagent (Herriott (1935–36)) One drop of copper sulfate solution is added to assure the maximum copper effect Less sodium hydroxide is added because there is less trichloracetic acid to neutralize When there is no acid other than the phenol reagent to be neutralized, 8 ml of sodium hydroxide is used in the colorimetric procedure

When cysteine is present, 1 ml of 38 per cent formaldehyde and 10 ml of 0 5 N sodium hydroxide are added to 5 ml filtrate and the phenol reagent is added 5 minutes later The formaldehyde diminishes the color due to the hemoglobin split products but almost abolishes the color given by cysteine As a control, when the effect of cysteine on digestion is being tested, the amount of cysteine present during digestion is added just before the addition of trichloracetic acid

Definition of Cathepsin Unit [C u][n]—The initial rate of digestion by 1 unit of cathepsin is such that there is produced per minute at 37°C in 6 ml of digestion mixture an amount of color producing substance not precipitable by trichloracetic acid which gives the same color as 1 milliequivalent of tyrosine

Calibration Curves—To 10 ml of hemoglobin solution are added 20 ml of trichloracetic acid and then 2 ml of enzyme solution The suspension is filtered To 5 ml of filtrate are added 1 ml of tyrosine solution, 10 ml of 0 5 N sodium hydroxide, and 3 ml of phenol reagent Three samples are treated in this way, the tyrosine solutions containing 0 075 0 15, and 0 225 mg tyrosine The colors are compared with the color given by 0 15 mg tyrosine dissolved in 8 ml of water plus 8 ml of 0 5 N sodium hydroxide When the results are plotted it will be found that they fall in a straight line which gives the relation between the color given by various amounts of tyrosine in pure solution and in the trichloracetic acid filtrate

Digestion is carried out for various lengths of time with 1 ml enzyme solution and the colors are read as previously described Doubling the time is taken to be equivalent to doubling the amount of enzyme From the straight line already obtained, the tyrosine equivalents of 5 ml samples of filtrate are read These values are converted into milliequivalents (0 15 mg = 0 00083 milliequivalent) and multiplied by 16/5 to give the tyrosine equivalents of the whole 16 ml of filtrate From such data the curve of Fig 1 is plotted

Recently, using cathepsin from spleen and using substrate and enzyme solutions having a very small blank I obtained a curve slightly different from the curve of Fig 1 For the present, therefore, the validity of the curve used for the estimation of any cathepsin should always be checked.

Water Blank—To obtain a water blank, 1 ml of water and 10 ml of trichlor

acetic acid are added to 5 ml of hemoglobin solution The color given by 5 ml of filtrate plus 1 ml of tyrosine solution containing 0 15 mg tyrosine is measured If this color value changes either a correction should be made or the hemoglobin solution rejected

If there is much difference between the water blank and the enzyme blank, the enzyme solution should be purified or diluted
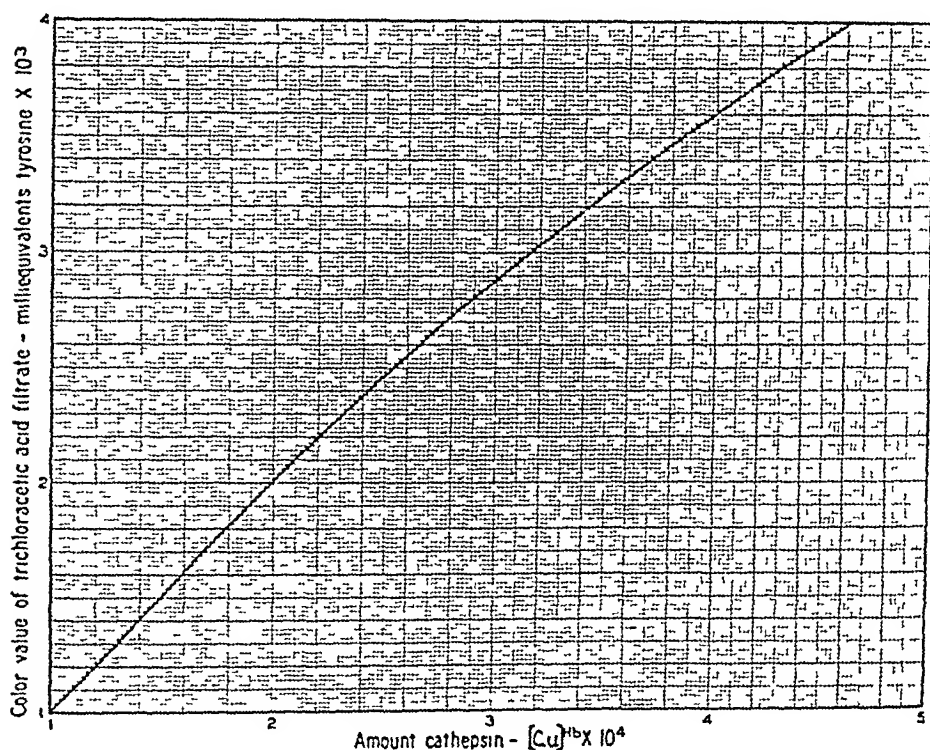
Fig 1 Relation of amount of cathepsin used for 10 minutes digestion of hemoglobin at 37°C and color value of trichloracetic acid filtrate

If there is not much difference between the water blank and the enzyme blank, calculations can be avoided by using a curve giving the cathepsin unit corresponding to various colorimeter readings

Effect of Various Substances on Digestion —The addition of 1 ml of the following solutions to 6 ml of digestion mixture has the same effect, within 3 per cent, as the addition of 1 ml water 0 1 N hydrochloric acid, 0 1 N sodium hydroxide, glycerine, 0 05 M ammonium sulfate, 1 N sodium chloride, 0 01 M iodoacetic acid, 0 02 copper sulfate, 1 M urea 1 ml 0 1 M cysteine has the same effect on the color value

of the trichloracetic acid filtrate in formaldehyde solution whether the cysteine is added before the enzyme or at the end of digestion just before the addition of trichloracetic acid

Effect of Temperature—The amount of digestion of hemoglobin produced by cathepsin in 10 minutes at 37°C is the same as that produced in 18 minutes at 25°C

Stability—The stability of cathepsin in the standard hemoglobin solution is shown by the experiments given in Table I   Were the enzyme not stable, there would be more digestion in 10 minutes by $3 8 \times 10^{-4}$ [C u]^{Hb} than in 60 minutes by one sixth that amount of enzyme

TABLE I

Stability of Cathepsin in Hemoglobin Solution

1 ml enzyme solution plus 5 ml hemoglobin solution — 25°C

Enzyme solution	Digestion	Color value 5 ml trichlor acetic acid filtrate
	min	*mg tyrosine*
(1) 1 ml cathepsin solution $3 8 \times 10^{-4}$ [C u]^{Hb}	10	0 198
(2) 1 ml cathepsin solution $3 8 \times 10^{-4}$ [C u]^{Hb}	60	0 193
(3) 1 ml digestion mixture (1) removed after 10 min at 25 C contains some split products	60	0 207

Destruction in Acid—The experiments given in Table II show that the addition of 0 5 ml of 0 4 N hydrochloric acid to 5 ml of hemo globin solution (pH of mixture, 2 3) causes a decrease in the rate of digestion which is due to destruction of the enzyme and that the rate of digestion in the hydrochloric acid hemoglobin solution is higher and the destruction of enzyme less if cysteine is added

Effects of Iodoacetic Acid and Cysteine on Digestion of Globin, Casein, and Gelatin as Measured by Trichloracetic Acid and Formol Titration Methods—The experiments given in Table II show that (1) the digestion of globin and casein, as measured by the trichloracetic method, is not affected by cysteine and iodoacetic acid, (2) the diges tion of globin, as measured by formol titration, is increased by cysteine and decreased by iodoacetic acid, and (3) in the presence of both

cysteine and iodoacetic acid, liver extract causes no increase in the formol titration of gelatin

The globin solution was a 2 per cent solution of acid acetone beef globin (Anson and Mirsky (1929–30)) The pH (by glass electrode) was 2 7

TABLE II

Instability of Cathepsin in Acid Solution Partial Protection by Cysteine

Enzyme solution	Substrate solution	Diges tion at 25°C	Color value 5 ml trichlor acetic acid filtrate	Color value 5 ml trichlora cetic acid filtrate + 1 ml form aldehyde
		min	*mg tyrosine*	*mg tyrosine*
(1) 1 ml cathepsin 2 3 × 10⁻⁴ [C u]ᴴᵇ	5 ml hemoglobin	10	0 137	0 093
(2) " "	5 ml hemoglobin + 0 5 ml 0 4 N hydrochloric acid	10	0 06	
(3) " "	" "	20	0 062	
(4) 1 ml digestion mixture (3) removed after 10 min	5 ml hemoglobin	60	Negligible	
(5) 1 ml cathepsin 4 6 × 10⁻⁴ [C u]ᴴᵇ	To 5 ml (5 ml hemoglobin + 0 5 ml 0 4 N hydrochloric acid) add 0 5 ml 0 1 M cysteine hydrochloric + 0 5 ml 0 1 N sodium hydroxide	10		0 111
(6) " "	Like (5) but cysteine and sodium hydroxide added just before trichloracetic acid	10		0 006
(7) 1 ml digestion mixture (5) removed after 10 min	5 ml hemoglobin	45		0 088
(8) 1 ml digestion mixture (6) removed after 10 min after addition of cysteine and sodium hydroxide	5 ml hemoglobin	45		0 055

The gelatin solution was a 2 5 per cent solution of isoelectric gelatin (Northrop and Kunitz (1927–28)) in water The pH of 10 ml gelatin solution plus 0 5 ml 0 2 M cysteine hydrochloride was 3 2

To prepare the casein solution 2 5 gm Hammarsten's casein and 15 gm urea

are shaken together and dissolved by the addition first of 18 ml 0 1 N hydrochloric acid and then of 82 ml water, pH by glass electrode, 3 2 With more or less urea the rate of digestion is less

TABLE III

Effect of Iodoacetic Acid and Cysteine on Digestion by Liver Cathepsin as Measured by Trichloracetic Acid and Formol Titration Methods

1 ml. cathepsin [C. u]Hb × 10⁴	Substrate solution	Digestion at 37 C.	Increase in formol titration of 6 ml digestion mixture	Color value 5 ml trichlor acetic acid filtrate	Color value 5 ml trichlor acetic acid filtrate + 1 ml form aldehyde
		min	ml 0 0 N sodium hydroxide	mg tyrosine	mg tyrosine
(1) 1 5	5 ml globin + 0 5 ml 0 1 M cysteine hydrochloride + 0 4 ml 0 1 N sodium hydroxide	20			0 115
(2) 1 5	Like (1) but cysteine and sodium hydroxide added just before trichloracetic acid	20			0 109
(3) 1 5	5 ml globin + 1 ml water	20		0 135	
(4) 1 5	5 ml globin + 1 ml 0 01 iodoacetic acid	20		0 135	
(5) 7 5	5 ml of (10 ml globin + 1 ml water)	20	0 65		
(6) 7 5	5 ml of (10 ml globin + 1 ml 0 01 iodoaceticacid)	20	0 45		
(7) 7 5	5 ml of (10 ml globin + 0 5 ml 0.2 M cysteine hydrochloride + 0 8 ml 0 1 N sodium hydroxide)	20	1 25		
(8) 3 75	5 ml casein + 1 ml 0 01 N hydrochloric acid	10		0 143	
(9) 3 75	5 ml casein + 1 ml 0 01 N iodoacetic acid	10		0 138	
(10) 7 5	5 ml of (10 ml gelatin + 0.5 ml 0 2 N hydrochloric acid)	20	0 05		
(11) 7 5	5 ml of (10 ml gelatin + 0 5 ml 0 2 M cysteine hydrochloride)	20	0 55		
(12) 7 5	5 ml of (10 ml gelatin + 0 5 ml 0 2 M cysteine hydrochloride + 0 2 ml 0 1 M iodoacetic acid)	20	0 05		

The trichloracetic acid procedure is that described for the hemoglobin method, the formol titration procedure that described by Northrop (1932–33)) except that the entire titration is carried out with 0 02 N sodium hydroxide

Preparation of Enzyme—Frozen beef liver is ground, stirred with twice its

weight of water and 15 per cent its weight of toluol (Eastman practical), allowed to stand overnight at room temperature, and filtered through gauze   1 N hydrochloric acid is then added with stirring, the proper amount being first determined by trial with a small sample of solution   If 55 gm of ammonium sulfate is added immediately per liter of acidified sample and the suspension filtered, the hemoglobin-free filtrate should be olive green to brom cresol green   An hour after the proper amount of hydrochloric acid has been added to the bulk of the solution 55 gm ammonium sulfate is added per liter of acidified solution   The precipitate formed is filtered off and the filtrate is made purple to brom cresol purple by the addition of 1 N sodium hydroxide   364 gm of ammonium sulfate is added to each liter of solution and the suspension is filtered   The precipitate is dialyzed overnight against cold 2 per cent sodium chloride in a shaking dialyzer   The amount of enzyme preparation used for estimation (10 minutes digestion at 37°C) has about 0 2 mg protein nitrogen   This has about the same activity as the autolysate from 70 mg of liver

The purification of cathepsin will be discussed in detail in a later paper   Since the experiments described in this paper were carried out, the procedure for the purification has been changed and the purification carried further   The specific activity of the present preparation is of the order of the specific activities of the crystalline proteinases   This very active cathepsin causes no appreciable increase in formol titration when added to an acid gelatin solution containing cysteine   Such a result was expected since the experiments described in this paper showed that the digestion of gelatin which can be measured by formol titration and which is increased by cysteine and decreased by iodoacetic acid is due not to cathepsin but to peptidases which accompany cathepsin in crude or partially purified extracts

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THE CALIBRATION OF DIFFUSION MEMBRANES AND THE CALCULATION OF MOLECULAR VOLUMES FROM DIFFUSION COEFFICIENTS

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INTRODUCTION

In the membrane method for measuring diffusion coefficients (Northrop and Anson (1928–29)) diffusion takes place through a porous disc. Under these conditions convection currents which usually make diffusion measurements difficult are avoided, the time required for a measurement is greatly shortened, and it becomes possible to remove the diffused substance for estimation. Under suitable conditions the rate of diffusion of one substance is independent of other substances present. To calculate the diffusion coefficient it is necessary to know only the per cent, not the absolute amount, of the original material which passes through the disc in a given time. The method can be applied, therefore, even to impure biological substances which can be estimated only by activity measurements. Since the dimensions of the pores are unknown, the membrane is calibrated by measuring the rate of diffusion through it of a substance of known diffusion coefficient.

The first part of this paper describes the calibration of diffusion membranes with sodium chloride, potassium chloride, and hydrochloric acid and summarizes the evidence that the membrane method yields correct diffusion coefficients. The second part of this paper states the assumptions made in calculating molecular volumes from diffusion coefficients by Einstein's law, states the consequences if these assumptions are not valid, and discusses the possible reasons why molecular volumes of various proteins calculated from their diffusion coefficients are higher than the molecular volumes calculated from osmotic pressures and sedimentation data. The third part

outlines the uses to which diffusion measurements can be put despite the limitations in the application of Einstein's law

Our original paper was primarily concerned with the technical problems involved in diffusion measurements and a detailed discussion of the limitations and possibilities in the use of the method was omitted   This omission gave rise, on the part of some wishing to use the method for biological materials, to a certain amount of confusion which we hope will now be avoided

1 The Validity of the Membrane Method for Measuring Diffusion Coefficients

Before discussing the validity of calculations made from diffusion rates measured by the membrane method we shall first state the evidence that the diffusion data themselves are correct   First, it has been shown by special experiments that there is no important disturbance from convection currents, that there is adequate stirring of the solutions above and below the membrane, and that the results are independent of the material, structure and, within certain limits, of the dimensions of the membrane (Northrop and Anson (1928–29)) Secondly, the assumption that there is a linear concentration gradient across the membrane, while not strictly correct, does not lead to any significant error (Barnes (1934))   Lastly, the diffusion coefficients of various salts and non-electrolytes and of hemoglobin are the same, within the experimental error, whether measured by the membrane method or by the classical method which does not involve the use of a membrane   Thus, the measurements given in this paper show that the membrane and the classical methods give the same results for the effect of temperature on the rate of diffusion of sodium chloride and for the ratio of the diffusion coefficients of sodium and potassium chlorides   Similar data can be found in the paper of McBain and Liu (1931)   The value of Northrop and Anson (1928–29) for the diffusion coefficient of 2 per cent hemoglobin at 5°C is 0 034 cm²/day (assuming the diffusion coefficient of 0 1 N hydrochloric acid at 5°C to be 1 45 cm²/day)   The value of Tiselius and Gross (1934) for 1 per cent hemoglobin at 20°C obtained by the classical method is 0 0542 cm²/day   Extrapolated to 5°C by Einstein's equation it is 0 0332 cm²/day   Lamm and Polson (1936) have recently obtained a value

for the diffusion coefficient of hemoglobin which is 9 per cent higher than that obtained by Tiselius and Gross As yet there has not been a sufficiently detailed study of the diffusion of any protein by both the classical and the membrane methods to prove that the two methods when applied to proteins yield exactly the same results In particular, the comparison of the two methods has not been made in any case in which the diffusion coefficient is known to be independent of concentration Most of the values of diffusion coefficients of proteins obtained by the membrane method, furthermore, were obtained from experiments incidental to other work, so that each value is based on only a small number of measurements

The Calibration of Diffusion Membranes

Sodium Chloride —Sodium chloride is a suitable substance for the standardization of diffusion membranes because its diffusion coefficient is not sensitive to concentration As measured by the membrane method at 5°C the diffusion coefficients of 0 2 N, 1 0 N, and 2 0 N sodium chloride are the same within the experimental error of 2 per cent In practice 2 0 N sodium chloride is used for calibration because the greater the amount of diffused sodium chloride available for estimation the easier and more accurate is the titration with silver nitrate When the diffusion coefficient is sensitive to concentration the results obtained by different methods are not comparable because the changes of concentration involved in the different procedures are different The differences in concentration changes are more serious the more concentrated the solution Very few of the diffusion coefficients in the literature are true diffusion coefficients, *i e* diffusion coefficients which really apply to the concentrations to which they are supposed to apply and are not averages of the diffusion coefficients corresponding to various concentrations

The rate of diffusion of 2 N sodium chloride has been measured by the membrane method at 5°, 10°, 18°, 20°, and 25°C See Table I The data fit within 2 per cent the straight line equation

$$\frac{D_{t_2} - D_{t_1}}{t_2 - t_1} = 0\ 0275 \text{ cm}^2/\text{day per degree}$$

$$t = C \tag{1}$$

Taking D to be 0 72 cm^2/day for $t_2 = 5$ (Oholm (1905)) equation (1) becomes

$$D = 0\ 588 + 0\ 0263\ t \tag{2}$$

On this basis $D_{18} = 1\ 06$ cm^2/day, which agrees within the experimental error with the results of Oholm (1905) and Clack (1917, 1921, 1924) See Table I It must not be assumed that the equation holds for temperatures lower than 5°C or higher than 25°C The data fit the Nernst (1888) equation [1]

The diffusion cells used in most of the measurements given in this paper were of about 50 ml capacity 50 ml of water was in outside vessels similar to the one pictured in the paper of Scherp (1932–33) The membranes were of Jena sintered glass,[2] porosity G 4, and about 4 cm in diameter and 1 5 mm in thickness At 25°C about 1 per cent of the sodium chloride diffused through the membranes in an hour

As an indicator for the titration of sodium chloride with silver nitrate, 4 drops of 5 per cent potassium chromate are added to 50 ml of solution and the titration

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[1] The temperature coefficient, α, of diffusion is usually defined as

$$\frac{D_{t_2} - D_{t_1}}{D_{t_2}} \quad \frac{1}{t_2 - t_1} = \alpha \qquad t_2 > t_1$$

The value of α as given by this equation is not the same for different temperature ranges unless the calculations are made from a constant D_{t_2} as a base If D_{t_2} is taken to be constant, the equation becomes identical with equation (1)

[2] Diffusion cells with fused in membranes can be obtained from Schott and Company, Jena, Germany (American agents, Fish-Schurman Corporation, New York City)

In the catalogue of the Jena Glass Company our diffusion apparatus is pictured in the form used by McBain This differs in two unessential details from the form now used by us First, the membrane is several times thicker than ours The thicker membrane may be an advantage for very accurate measurements of the rate of diffusion of small molecules When the rate of diffusion of slowly diffusing biological molecules is being measured thinner membranes which permit more rapid diffusion are more desirable Secondly, we have the stop-cock immediately above the wide part of the diffusion cell, whereas McBain has it higher on the tubing attached to the wide part, as in our original apparatus The purpose in placing the stop-cock lower is to avoid a dead space in which convection currents may not produce adequate stirring The manufacturers will make diffusion cells of any desired design and dimensions

is carried to a definite brown Since the greater the amount of silver chloride in suspension the more silver nitrate is needed to turn the indicator brown, known amounts of sodium chloride are titrated to obtain a calibration curve

Potassium Chloride —McBain and his associates have used our membrane method extensively They calibrate their membranes with 0 1 N potassium chloride at 20°C taking for the diffusion coeffi-

TABLE I

Diffusion Coefficients at Various Temperatures — Cm²/Day

	5	10°	12	16	18°	20°	25
2 N sodium chloride	0 720	0 848			1 04	1 12	1 27
	(0 720)	(0 851)			(1 06)	(1 11)	(1 25)
1 N sodium chloride (Oholm)	0 720				1 06		
2 N sodium chloride (Scheffer)	0 72						
1 N sodium chloride (Clack)					1 04		
					1 07		
0 5 N potassium chloride							1 56
0.5 N potassium chloride (McBain and Dawson)							1 57
1 N hydrochloric acid	1 60	1 85	(1 96)	2 16	(2 27)		2 65
	(1 59)	(1 85)		(2 17)			(2 64)
1 N hydrochloric acid (Oholm)			1 96				
0 4 — 0 5 N hydrochloric acid (Scheffer)	1 55	1 78					
0 1 N hydrochloric acid	1 45	1 68	(1 77)	1 94			2 38
	(1 45)	(1 68)		(1 96)			(2 37)
0 1 N hydrochloric acid (Oholm)			1 98	2 13			

The values are diffusion coefficients expressed in cm²/day

The values in parenthesis are calculated from the equations given in the text

The membranes were calibrated with 2 N sodium chloride, taking D_5 = 0 72 from Oholm

cient the accurately determined value of Cohen and Bruins (1924), 1 249 cm²/day The diffused salt is estimated with an interferometer 0 1 N potassium chloride has two disadvantages as a substance for calibration First, the accurate estimation of very small amounts of potassium chloride is, in the absence of suitable optical equipment, not convenient Secondly, according to McBain and Dawson (1935) the rate of diffusion of dilute potassium chloride is not independent of the concentration so that the results obtained by the membrane method

are not strictly comparable to those of Cohen and Bruins The possible error here is only a few per cent

Using membranes calibrated with 0 1 N potassium chloride McBain and Dawson (1935) found the diffusion coefficient of 0 5 N potassium chloride at 25°C to be 1 57 cm²/day Using membranes calibrated with 2 N sodium chloride, we find the diffusion coefficient of 0 5 N potassium chloride to be 1 56 cm²/day

Hydrochloric acid —The rate of diffusion of 0 1 N and of 1 0 N hydrochloric acid through membranes calibrated with 2 N sodium chloride has been measured at 5°, 10°, 16°, and 25°C The results (Table I) within 1 per cent are expressed by the equations

$$D_{0\ 1N\ HCl} = 1\ 22 + 0\ 046\ t$$
$$D_{1\ 0N\ HCl} = 1\ 33 + 0\ 0525\ t$$

Our results with 1 N hydrochloric acid agree with Oholm's Our results with 0 1 N hydrochloric acid do not Oholm's value for the diffusion coefficient of 0 1 N hydrochloric acid is 11 per cent higher than ours at 12°C , 9 per cent higher at 16°C

Scheffer measured the diffusion coefficient of 0 5 N hydrochloric acid at 5°C and 10°C His values are between our values for 0 1 N hydrochloric acid and 1 N hydrochloric acid See Table I

It should be pointed out that if the diffusion coefficient of hydrochloric acid is dependent on the concentration, then the results obtained by Oholm's method and the membrane method are not strictly comparable The deviation from the true diffusion coefficient is more serious when Oholm's method is used Furthermore, Oholm regarded his experiments with hydrochloric acid as less accurate than those with sodium chloride In his more concentrated acid solutions visible precipitates were formed by the reaction of the acid with the mercury supporting the solutions

Correction of Old Calibration —The diffusion coefficient of 0 1 N hydrochloric acid at 5°C , as measured with a membrane calibrated sodium chloride, is 1 45 cm²/day In our original use of the membrane method we calibrated our membranes with 0 1 N hydrochloric acid at 5°C and took the diffusion coefficient as 1 85 cm²/day This value was obtained by extrapolation from Oholm's data Zeile (1933) pointed out that we made a numerical error in calculation and that

the effect of temperature on the rate of diffusion of hydrochloric acid is greater than that given by Oholm We have now found, in addition, a discrepancy between Oholm's absolute values and the absolute values obtained by the membrane method with membranes calibrated with sodium chloride Since from the data in the literature, one cannot be certain what the absolute diffusion coefficient of hydro chloric acid is at any given temperature or what the effects of tempera ture and concentration are on the diffusion coefficient, it is clear that hydrochloric acid is not at present a suitable fundamental standard for the calibration of diffusion membranes

The Assumptions Involved in the Calculation of Molecular Weights from Diffusion Coefficients

Although the measurement of rates of diffusion by means of the membrane method is simple, the calculation of molecular weights from diffusion constants involves many uncertainties These uncertainties have nothing to do with the validity of the diffusion data themselves They are, for the most part, independent of the technique used in making the diffusion measurements According to Einstein (1908) the diffusion coefficient D is related to the friction coefficient F as follows

$$D = \frac{RT}{N} \; \frac{1}{F} \tag{1}$$

This equation states that the rate of diffusion of a substance from one part of a solution to another is directly proportional to the difference in osmotic pressure due to the difference in concentrations of the sub stance in the two parts of the solution, and inversely proportional to the coefficient of friction, which is the force needed to produce unit rate of motion The assumptions made are (1) that D is a true diffusion constant independent of the concentration of the diffusing substance, (2) that van't Hoff's osmotic pressure law is obeyed, which again means that D is independent of the concentration of the diffus ing substance, and (3) that D is not influenced by other diffusing substances The diffusion of protein from hydrochloric acid solution into water, for instance, is much faster than the diffusion of isoelectric protein into water The small negatively charged chloride ions diffuse

rapidly and drag with them the large protein ions of opposite charge from which they cannot be separated   This accelerating effect can be abolished by salt just as Donnan effects can be abolished in osmotic pressure experiments   If diffusion experiments are carried out with dilute solutions containing salt, and if D is independent of the concentration of the diffusing substance and of the salt, then the assumptions made in deriving equation (1) may be considered as experimentally justified and F may safely be calculated from D

Einstein takes F to be related to the radius of the diffusing particle r and the viscosity of the solvent, η, by Stokes' law

$$F = 6\pi r\eta \tag{2}$$

Combining equations (1) and (2) one obtains

$$D = \frac{RT}{N} \; \frac{1}{6\pi r\eta} \tag{3}$$

which is Einstein's law   The assumptions involved in Stokes' law are that the diffusing particles are large and few in number in comparison with the molecules of the solvent and that they are spherical

If the diffusing particles are not spherical the calculated radius is too great   It is impossible to find out from diffusion experiments alone whether the diffusing particles are spherical or how great an error is made in making the assumption that they are spherical Theoretical calculations have been made for particles of certain definite shapes (Svedberg (1928))   For instance, the coefficient of friction of an ellipsoid whose long axis is ten times its short axis is about 20 per cent greater than the coefficient of friction of a sphere of the same volume

Finally, the friction which, other things being equal, determines the rate of diffusion is the friction between the whole kinetic unit and the solvent   If the diffusing molecule is hydrated then the radius given by Einstein's law is the radius of the hydrated molecule and the molecular volume calculated from the radius is the volume of the hydrated molecule   In contrast, osmotic pressure measurements yield information about the volume of the unhydrated molecule There is no way of telling from diffusion experiments alone whether a molecule is hydrated or not, or to what extent it is hydrated

If the diffusion has been accelerated by ionic effects then the calculated radius is too low   This acceleration is easily abolished by salt If, on the other hand, any of the other assumptions made in arriving at Einstein's law is not justified in any particular case, then the calculated radius is too high

We have seen that some of Einstein's assumptions, in particular those involved in the equation relating D to F, can be tested experimentally by diffusion measurements   Others cannot   Even if all the possible tests have been carried out, the molecular weight of the unhydrated molecule calculated from D may be higher than that obtained from osmotic pressure data because the molecule is either hydrated or non spherical or both

Comparison of Molecular Volumes Calculated from Osmotic Pressures, Sedimentation Data, and Diffusion Coefficients

Table II shows that the volumes of various protein molecules calculated from osmotic pressure and sedimentation data agree but are lower than the volumes calculated from diffusion coefficients   The volumes calculated from osmotic pressure and sedimentation data do not include any water of hydration   The calculations do not involve any assumptions about the shapes of the molecules   The volumes calculated from diffusion data, as we have seen, do include water of hydration and are based on all the assumptions involved in Einstein's law   There are three possible reasons why the volumes calculated from diffusion coefficients are higher than those from osmotic pressure

1 The diffusion coefficients are too low   We have already presented the evidence for the correctness of the diffusion coefficients

2 The molecules are hydrated and the difference between the volume calculated by the other two methods represents water of hydration   This hypothetical water of hydration, however, is higher than the hydration of these proteins calculated from viscosity data by the empirical equation of Kunitz (1925–26)   This disagreement is not conclusive proof that hydration is not responsible for the high volumes calculated from diffusion coefficients because the application of Kunitz's equation may not be valid

In a previous paper (Kunitz, Anson, and Northrop (1933–34)) the

TABLE II

Molecular Volumes

	Calculated from osmotic pressures	Calculated from sedimentation data	Calculated from diffusion coefficients
Hemoglobin	50,200 (Adair (1925))	52,000 (Lamm and Polson (1936))	104,500* (Northrop and Anson (1928–29))
Pepsin	26,300 (Northrop (1929–30))	26,000 (Philpot and Eriksson-Quensel (1933))	56,000* (Northrop (1929–30))
Trypsin	27,500 (Kunitz and Northrop (1934–35))		92,400* (Scherp (1932–33))
Chymo trypsinogen	17,100 (Kunitz and Northrop (1934–35))		56,100† (Kunitz and Northrop (1934–35))
Chymo trypsin	30,700 (Kunitz and Northrop (1934–35))		56,100† (Kunitz and Northrop (1934–35))

* Membrane calibrated with 0 1 N hydrochloric acid — $D_5° = 1\ 45$ cm²/day
† Membrane calibrated with 0 23 N sodium chloride — $D_5° = 0\ 72$ cm²/day

hydration calculated from viscosity data was found to agree with the difference between the volumes calculated from diffusion coefficient and osmotic pressure This result must be rejected, however, because the diffusion coefficients used were based on a wrong value for the diffusion coefficient of 0 1 N hydrochloric acid at 5°C

3 Einstein's equation is not valid

(a) The relation between the coefficients of diffusion and friction

$$D = \frac{RT}{NF}$$

is not correct when applied to proteins Lamm and Polson (1936), however, have shown that the molecular weight calculated from sedimentation equilibrium agrees with the molecular weight calculated from sedimentation velocity using the coefficient of friction obtained from the diffusion coefficient This agreement would not exist were not the coefficient of friction calculated from the diffusion coefficient correct

(b) Stokes' law

$$F = 6\pi r \eta$$

is not valid when applied to protein molecules As we have already pointed out, if the molecule is not spherical the radius and hence the volume calculated from the diffusion coefficient by Einstein's law which includes Stokes' law is too high

Polson (1936) has assumed that the molecular weights calculated from diffusion coefficients are higher than those calculated from sedimentation data because the molecules are ellipsoids and not spheres, and that corrections for the non sphericity of the molecules can be calculated from viscosity data, using the equations of Kuhn and Arrhenius When such corrections were made he found that the molecular weights of a number of proteins calculated from diffusion and viscosity data were about 70 per cent of those calculated from sedimentation data

(c) Einstein assumes that the viscosity, η, in Stokes' equation is the viscosity of the solvent and that the viscosities of the solvent and the solution are the same We have used the viscosity of the solvent in calculating the molecular volume from the diffusion coefficient but

the viscosities of the solutions were, in general, higher than the viscosities of the solvents The volumes calculated from diffusion coefficients, however, would still be high even if the viscosities of the solutions were used in the calculations

In general, it may be said that the molecular volumes calculated from diffusion coefficients are higher than those calculated from osmotic pressure and sedimentation data, but that it cannot be decided whether these high values are due to hydration or non-spherical shape or both because there is no reliable information at present about the hydration and shapes of protein molecules

The Kinds of Information Which Can Be Obtained from Diffusion Measurements

Since Einstein's law involves assumptions which in practice may not be valid, there is some doubt as to the significance of the molecular volumes calculated from diffusion coefficients Useful information, however, can be obtained from the diffusion measurements despite this limitation

In the first place, by assuming Einstein's law to be correct one can obtain a rough notion of the maximum molecular size from a knowledge of the diffusion coefficient The membrane method, in fact, was originally devised to obtain rough information about molecular size of biologically active substances when the ordinary methods of measuring molecular size cannot be applied at all By means of the membrane method one can measure the diffusion coefficient of, for instance, bacteriophage whose presence is known only by its biological activity and which is available only in low, unknown absolute concentration in impure solution (Hetler and Bronfenbrenner (1930–31))

In the second place, by making only assumptions which can be tested experimentally, one can calculate the coefficient of friction, F, from the diffusion coefficient, D Knowing F one can calculate the molecular weight of a particle from the rate at which it moves in a gravitational field, as has been done by Svedberg, or the charge on a particle from the rate at which it moves in an electrical field, or in general, add to the information which can be obtained from a knowledge of the rate at which a particle moves under the influence of a given force

In the third place, several different kinds of useful information can be obtained from suitable diffusion experiments without any calculation of friction coefficient or molecular size, without any assumptions whatever about the shape of the molecule or its degree of hydration First, diffusion experiments can, under suitable conditions, give some information about the constitution of a substance If the rate of diffusion of a substance in the absence of salt is not affected by acid or base, then the substance is a non-electrolyte If the rate of diffusion is at a minimum at a given pH and is increased by both acid and base, then the substance is amphoteric Secondly, diffusion experiments can be used to test the homogeneity with respect to particle size of the diffusing substance If the first part of the substance which has diffused through the membrane is removed and the rate at which this diffusate diffuses through a membrane is measured in a separate experiment, and if the diffusion coefficient of the first diffusate is the same as that of the original substance, then the original substance is homogeneous Thirdly, when an active material has been supposedly isolated diffusion experiments can be used to test whether the substance isolated is actually the active substance If the active substance and the isolated substance are of similar size and constitution, then the rate of diffusion under all circumstances, even in the absence of salt and in the presence of acid or base, should be the same whether the amount diffused is measured by activity measurements or by direct estimation of the substance, for instance, by nitrogen determination

Finally, by means of diffusion substances which diffuse at different rates can be separated Large differences in rate of diffusion may exist between two substances of similar size if one substance is ionized and the other is not

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ACTION OF ULTRAVIOLET LIGHT ON SPORES AND VEGE-TATIVE FORMS OF B MEGATHERIUM SP

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These experiments have been made to determine the survival ratios of vegetative forms and spores of *B megatherium sp* under the influence of monochromatic ultraviolet light   As such they are an extension of previous irradiation studies made in this laboratory

Technique

A strain of *B megatherium sp* has been used which, besides being an exceptionally good spore former, produces vegetative forms that hold together only weakly in chains   Microscopic examination demonstrated that these chains could be broken up by moderate shaking to furnish the suspension of single organisms essential to good quantitative studies   The bacteria were cultivated at 25°C and stock cultures for spores were incubated at 37 C for 2 months in sealed tubes   The experimental methods for irradiation were similar to those previously described,[1] the bacteria being irradiated after spreading on an agar surface   To do this 1 cc of a 24 hour old broth culture was diluted with 10 cc of nutrient broth and left 60 minutes in 26°C   This fresh culture was then diluted ten times with saline, shaken mechanically for 10 minutes, and spread in 0 5 cc portions on nutrient agar, poured 24 hours previously into 7 5 cm Petri dishes   This bacterial suspension was left on the agar for 3 minutes, drained for 10 minutes at the room temperature, and put immediately in the ice box

The old cultures containing spores were placed for 10 minutes in a 60°C water bath before dilution, it having been found that this time and temperature were sufficient to kill all vegetative forms   The suspension was then diluted tenfold with saline, shaken for 10 minutes, and spread in the manner just described

After irradiation the plates were put in a thermostat at 14 C   This low incubation temperature was needed to give single colonies   At higher temperatures long chains of bacteria are formed which, spreading widely over the agar, give a confluent growth before the colonies can be counted

The arrangement for irradiation was the same as that previously used [1]   The source was a powerful quartz mercury arc, the monochromator was a large one,

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[1] Wyckoff, Ralph W G , *J Gen Physiol* , 1931–32, **15**, 351

having 6 inch fused quartz lenses and prisms   The current for the lamp, which was operated at either 100 or 119 volts, was drawn from a 200 volt storage battery of large capacity   Irradiations were made only after the arc had been running for at least 2 hours   Its output was controlled by an attached quartz sodium photoelectric cell   Single spectral lines were selected and the monochromator was adjusted till the irradiation slit, 29 x 3 mm , was uniformly filled with light of one wave length   The sharp edges of the slit were used to mark the irradiated and standard areas on the surface of the agar, several areas being irradiated on a single plate   Before and after each experiment the energy flux at the point of irradiation was measured by a thermocouple calibrated with a carbon lamp standardized by the U S Bureau of Standards   Survival ratios were determined from counts after incubation of the number of colonies on the irradiated and control areas

TABLE I

Survival Ratio of Vegetative Forms of B megatherium sp

Time	Wave length		Time	Wave length
	2536 Å	2803 Å		3132 Å
sec			*min*	
5	0 873	0 819	1	0 947
10	0 800	0 683	2	0 689
20	0 729	0 586	3	0 650
30	0 692	0 455	4	0 582
40	0 609	0 371	5	0 597
60	0 346	0 232	6	0 476
80	—	0 130	8	0 317
Energy incident per mm ^{2} per sec	2 9 ergs	5 5 ergs		64 0 ergs

EXPERIMENTAL RESULTS

The vegetative forms and spores of *B megatherium* were irradiated by light of the wave lengths 2536 Å, 2803 Å, and 3132 Å   The results are tabulated in Tables I and II and graphically represented by Figs 1 and 2   The survival ratios are averages of many counts, the mean number of control colonies for every point being 1580 Large counts are essential, especially for small survival ratios after prolonged irradiations, when the variations in the number of survivors influence the ratio very strongly

TABLE II

Survival Ratio of Spores of B megatherium sp

Time	Wave length	
	2536Å	2803 Å
sec		
30	0 814	0 689
40	—	0 773
60	0 700	0 546
90	0 397	0 304
120	—	0 306
180	0 296	0 076
240	0 209	0 051
Energy incident per mm.² per sec	2 6 ergs	5 7 ergs
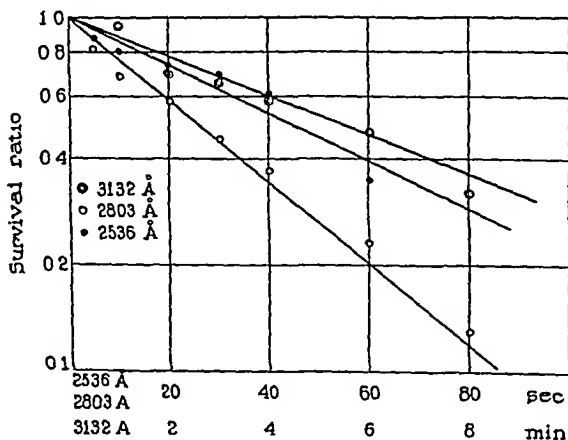
FIG 1 Survival ratio of the vegetative forms of *B megatherium sp* irradiated with ultraviolet light of wave lengths 2536 Å 2803 Å, and 3132 Å.

From the figures, it is evident that within the limits of experimental error (which amounts to *ca* 10 per cent) the results on both vegetative forms and spores are semilogarithmically linear   This conclu-
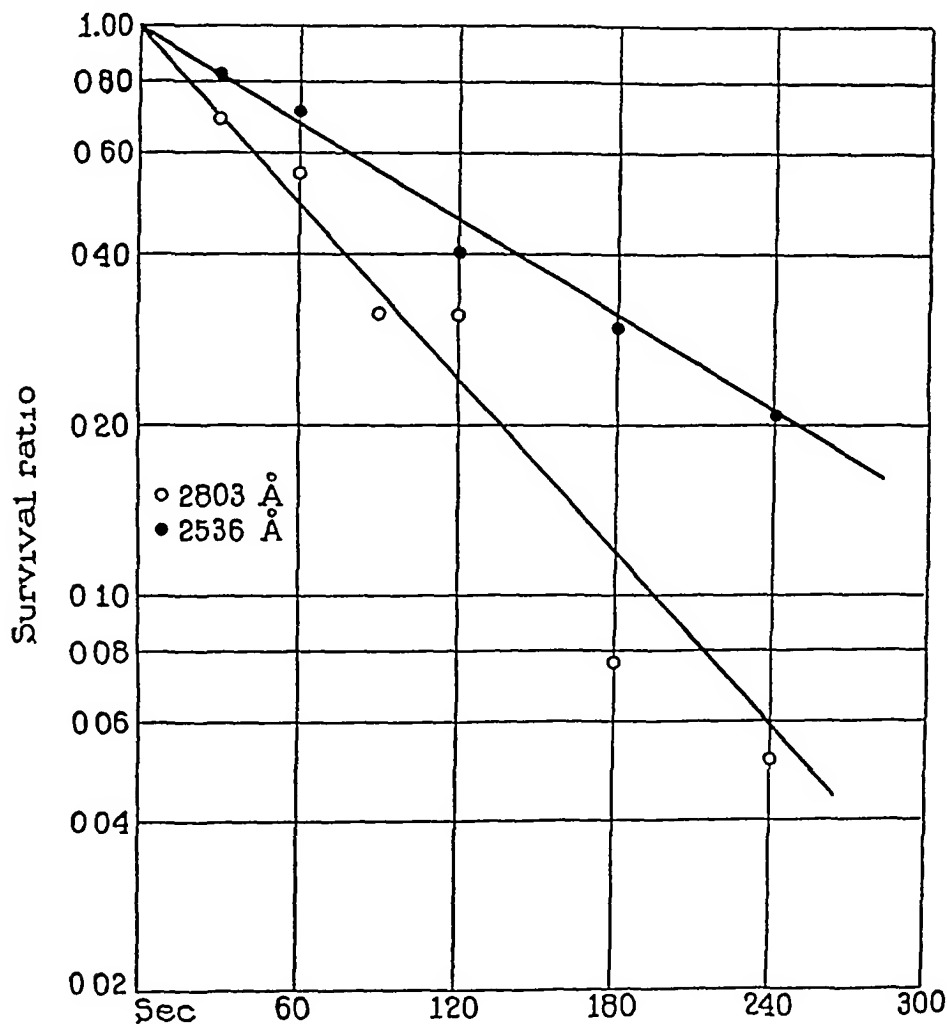
FIG 2 Survival ratio of the spores of *B megatherium* irradiated with ultraviolet light of wave lengths 2536 Å and 2803 Å

sion, though agreeing with the data on most other bacteria, conflicts with some recent experiments[2] in which it was found that the vegetative forms and spores of *B subtilis* and the spores of *B megatherium*

[2] Duggar, B M , and Hollaender, A , *J Bact* , 1934, 27, 241

sp have the survival ratios following a multiple hit curve   These results showed a wide range of variation, however, and it seems probable that single cell suspensions were not obtained

Incident energies necessary for 50 per cent killing (Table III) were calculated by multiplying the incident energies per second by the time of irradiation giving the survival ratio 0 5   Spores evidently are destroyed by about twice as much energy as is required for the vegetative forms   Sensitivities are of the same order of magnitude as those prevailing with *B coli* except that the 3132 Å energy is much greater for *B megatherium*   It would be interesting to determine from spectrophotometric studies whether this was due to differences in absorption spectrum

TABLE III

Energies for 50 Per Cent Killing of Bacteria

Wave length	B coli[1]		B megatherium		
	Incident energy	Energy absorbed per bacterium	Vegetative forms		Spores
			Incident energy	Energy absorbed per bacterium	Incident energy
	ergs/mm²	*ergs*	*ergs/mm²*	*ergs*	*ergs/mm²*
2536 Å	200	2 75 × 10⁻⁶	113	6 2 × 10⁻⁶	273
2803 Å	240	2 50 × 10⁻⁶	149	5 9 × 10⁻⁶	342
3132 Å	5200	10 9 × 10⁻⁶	21,150	18 × 10⁻⁶	—

By making the assumption, which may or may not be true, that the spectra of *B megatherium* and *B coli*[1] are the same, an estimate can be formed of the energy absorbed per bacterium for 50 per cent death   The results, computed on the basis of measurements indicating that the average bacillus irradiated is a rod 2 2μ long and 0 9μ in diameter, are listed in Table III   Ultraviolet microphotography[4] points to a very different absorption for spores, therefore no effort has been made to calculate the energy absorbed in them

SUMMARY

Spores and vegetative forms of a strain of *B megatherium* were irradiated by ultraviolet light of the wave lengths 2536 Å, 2803 Å,

[3] Gates, F L , *J Gen Physiol* , 1930-31, 14, 31

[4] Wyckoff, Ralph W G and Ter Louw A L , *J Exp Med* , 1931, 54, 449

and 3132 Å   The killing rate of both bacteria and spores is exponential, in agreement with irradiation results on other bacteria Twice as much incident energy is needed to kill the spores as the vegetative forms (50 per cent death)

The absorbed energy per bacterium for 50 per cent killing has been calculated on the assumption that the absorption of the vegetative cells is the same as that of colon bacilli   These results are compared with previous measurements on other bacteria

I am indebted to Dr R W G Wyckoff for his kind interest in this work

CONCERNING CRITICAL PERIODS IN THE LIFE OF ADULT DROSOPHILA

By W J CROZIER and E V ENZMANN

(From the Biological Laboratories, Harvard University, Cambridge)

I

To provide an explanation for systematic fluctuations in the re sistance of *Drosophila* to alcohol with advancing age it was suggested (Crozier, Pincus, and Zahl (1935–36)) that periodic metabolic ad justments occur at fairly definite ages, in genetically uniform stock under reasonably constant conditions   They might be said to mark *metabolic instars*   These adjustments were conceived to have the character of "suppressed moults," or rather of ecdyses which do not materialize,—in other words, to be of the nature which in Orthoptera for example result in successive ecdyses   It was pointed out that the life duration data on inbred lines of *Drosophila* indicated that the death rate appears to pass through successive accelerations at ages which differ in the various stocks and which are also influenced by sex   The sharpness of appearance of such recurrent periods of ac centuated susceptibility should be blunted or obscured by lack of real genetic uniformity in a stock considered, and in a life duration experiment their exhibition should vary with the severity of the conditions affecting survival

The reality of such critical metabolic conditions, superimposed upon the fundamental progression toward senility, can be tested by examining the rate of occurrence of deaths in the stock we have already tested for resistance to alcohol   The chief evidence for periodic changes in resistance to the toxic action of alcohol was de rived from the slopes of the straight lines relating survival time to vapor pressure of alcohol (Crozier, Pincus, and Zahl (1935–36)) The reciprocals of these slopes, taken as appropriate measures of resistance to diffusive penetration of alcohol, are related to age in

595

such a way that the resistance increases with age but on a curve for which the first derivative (i e , the rate of change of resistance) shows maxima at several ages, with intervening minima, differing for the two sexes   These maxima coincide with maxima of change in the vapor pressures of alcohol independently calculated to kill instantly   Maxima and minima of changing resistance to alcohol should be expected to show some definite correspondence with minima and maxima in the curve of rate of occurrence of deaths in a survivorship experiment with the same stock grown under the same reasonably constant conditions

<div align="center">II</div>

The stock of *Drosophila* used was started from a Florida wild type strain given us by Dr Demerec and maintained in inbred condition for several years under uniform conditions of culture at 25–26°C (*cf* Crozier, Pincus, and Zahl (1935–36))   We shall refer to this as line *F*   Our stock contains a recessive white-eyed mutation which apparently arose some time before our experiments were performed

Females were permitted to lay eggs for about 3 hours on a drop of culture medium on a 5 cm glass square   Drops were then peeled off and transferred to culture bottles (half-pint bottles), or the flies were permitted to deposit eggs for about 3 hours in new cultures   The culture medium contained 40 gm agar, 200 gm cornmeal, 280 cc molasses, 2000 cc water (or enough to give such a consistency that the medium settled in 15 minutes)   Each culture bottle received 1½ cm depth of the medium, and after cooling was inoculated with 2–4 drops of a 20 per cent yeast suspension and left standing overnight   A square of filter paper on top of the culture medium prevented accumulation of excess moisture

Newly hatched unmated flies were etherized, the sexes assorted, and transferred to empty bottles until recovery from the ether   35 to 60 individuals were placed in a single culture bottle for the determination of death rates

At 26°C the minimum time from egg laying to emergence of flies is 9 days   The culture media were changed to prevent possible contamination from accidentally fertilized flies   Bottles were changed routinely at intervals of 5–7 days, or when the medium became too moist, or when the mortality was unusually high and counting became difficult

The experiment was made in such a way that not all the bottles of the total population examined were initiated on the same day, but over a period of a week   This was done in order to make certain that no systematic rhythm of changing culture bottles could influence the occurrence of deaths in a systematic way   When the numbers of individuals in the bottles fell to 35 or less, the population in several bottles was combined so as to maintain the population in a single bottle between 35–45 at all times, the intention was to remove the influence of a density of population factor upon occurrence of deaths

The first experiment was made with 971 females and 962 males On its com pletion the whole experiment was repeated with 938 females and 898 males Dead flies were counted each day, at about the same time Agreement in the results of such repetitive tests is of course more significant than would be the increased formal precision to be obtained by enlarging the total numbers in any one run

In addition, we have made runs with mixed populations of strain F, and also with an eosin stock which has been inbred in this laboratory In a mixed popu lation of males and females, where the proportion of the two fluctuates the tem porary accelerations of the death rate are largely obscured

III

The running totals of observed deaths at successive ages $(\Sigma d'_x)$ are plotted in Fig 1 It is apparent that the rate of occurrence of deaths is not uniform It is also clear that the peculiarities of the curve for each sex are specifically exhibited in repetitions of the experiment The d'_x's for the two experiments have been added for purposes of further treatment of the curves for the two sexes The changes in rate of change of the death rate and the participation of a sex factor are of the type already indicated in previously available data upon other inbred lines of *Drosophila* (cf Crozier, Pincus, and Zahl (1935–36))

From measurements with line F of the time to-death in various partial pressures of alcohol vapor an invasibility coefficient S was calculated for flies of different ages (Crozier, Pincus, and Zahl (1935–36)) S was found to have a rather complex relationship to age of fly, and to differ for the two sexes The data are reproduced in Fig 2 Changes in the shape of the S age curve were found to be correlated with fluctuations in the vapor pressure P_0 of alcohol com puted to kill instantly Alterations in $-dS/dt$ were also found to be associated with maxima in the variation (σ_{lt}) of time to-death

We may regard the rate of change of S as an index of the rate of underlying metabolic events To make the notion specific, the increase of resistance to diffusive penetration of alcohol from its vapor, with advancing age of fly, may be conceived due to the super ficial deposition of cuticular materials, or to the progressive removal of materials favoring penetration, this being a result of general meta bolic events Alterations in $-dS/dt$ should therefore be correlated

with *changes* in the rate of progression of the underlying metabolic processes   Where dS/dt changes least rapidly, P_0 is at a maximum Where dS/dt exhibits rapid changes, P_0 is at a minimum   The determinations of S were based upon observations with flies of ages spaced at intervals of 1 day from 1 to 10 days inclusive, at intervals of 5
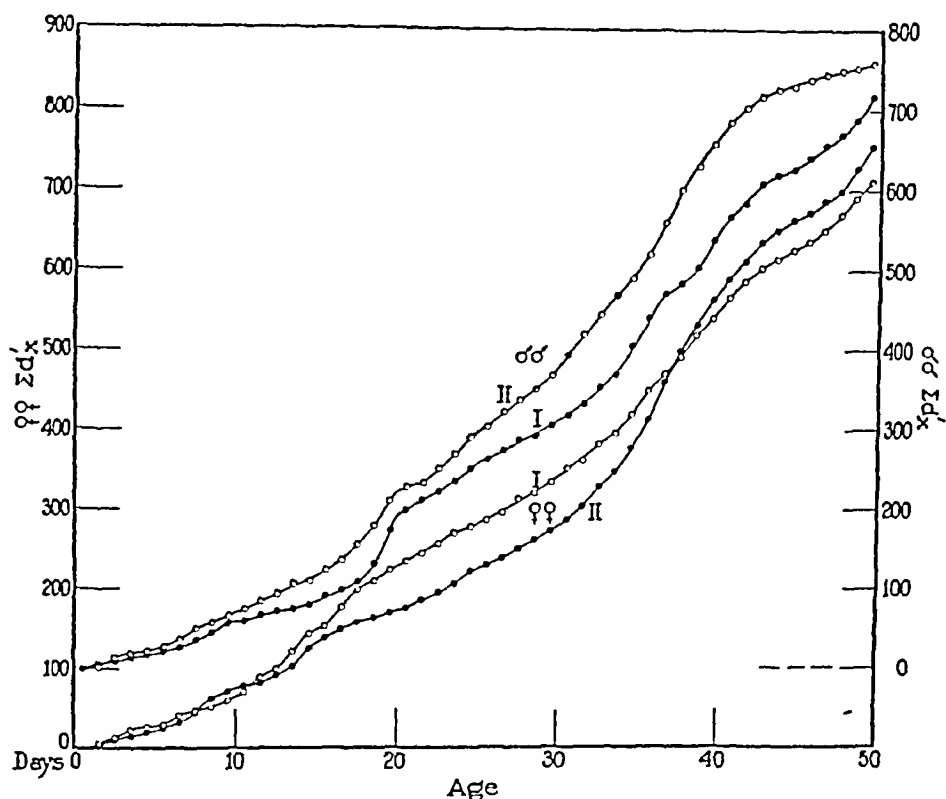
Fig 1 The summated number dead as a function of age in days at death, for ♂ ♂ and ♀ ♀ populations of *Drosophila*   The results in two separate experiments show corresponding accelerations for each sex   (For ♂ ♂, the origin has been raised—scale at the right)

days thereafter up to 50 days   The death rate data were obtained for intervals of 1 day throughout, we might thus reasonably expect greater complexity in the latter   Nonetheless, if ages of minimum and maximum resistance to the penetration of alcohol and to its toxic action are determined by the incidence of surges in metabolic progress

toward death, then there should be apparent rather definite correspondences between rate changes in $-dS/dt$ (i e, in d S/dt^2) and in the rate of accumulation of deaths   These maxima are deducible from Fig 3 as easily as from tabulated daily death rates (q'_z), which have been computed, the fluctuations are statistically significant, as well as being essentially coincident (for each sex) in independent repetitions of the experiment   It is also true that minima in q'_z coincide with regions of the S-age curve which show practical constancy of dS/dt These correspondences are perhaps most simply made clear by a plot of log $(1/\Sigma d'_z)$ as a function of age   Correspondences of changes in the slopes of this curve with those of the curve of S, as a function of age, would mean that ΔS per unit time (under constant conditions) has the same kind of meaning, so far as the aging function is concerned, as $d'_t/\Sigma d'_t$, d'_t signifies the number dying in 1 day at age t and $\Sigma d'_{zt}$ is the total dead up to and including that day—in other words, the percentage of the total deaths (up to that point) occurring at that particular age   The curves are given in Fig 3, where log $(1/\Sigma d'_z)$ is plotted against age   The correspondence with Fig 2 may fairly be considered close, if not indeed remarkable   The slope of the log $(1/\Sigma d'_z)$ curve is $-\Delta(\Sigma d'_z)/\Delta t \Sigma d'_z$, this has been assumed to be parallel to $-\Delta S/\Delta t$ With $\Delta t \approx 1$ day, this is identical with the statement that $d'_z/\Sigma d'_z \propto dS$ Examination shows that within each of the intervals between sharp changes in the slopes of the curves in Figs 2 and 3 there is a rectilinear relationship between log $(1/\Sigma d'_z)$ and S, but that the proportionality constant changes size from one such interval to another

The alcohol experiments demonstrate that *all* the individuals of one sex of the inbred strain F pass through critical periods at the same ages   The incidences of deaths under the conditions of the present tests show merely that the occurrence of the termination of life is increasingly probable at the particular intercycle ages   It is entirely justifiable to conclude that under uniform conditions of greater severity (*e g*, less food, more crowding, higher temperature) the changes of curvature in the $\Sigma d'_z$ curves would be more accentuated

The evidence shows that at these ages the resistance to alcohol (P_0) is at a minimum, that the rate of decrease of invasibility by alcohol vapor is changing most rapidly, and that the death rate goes

through a maximum   We may consequently take these particular ages as defining the time limits of metabolic instars, under the present conditions   It should be of interest to ascertain the manner of
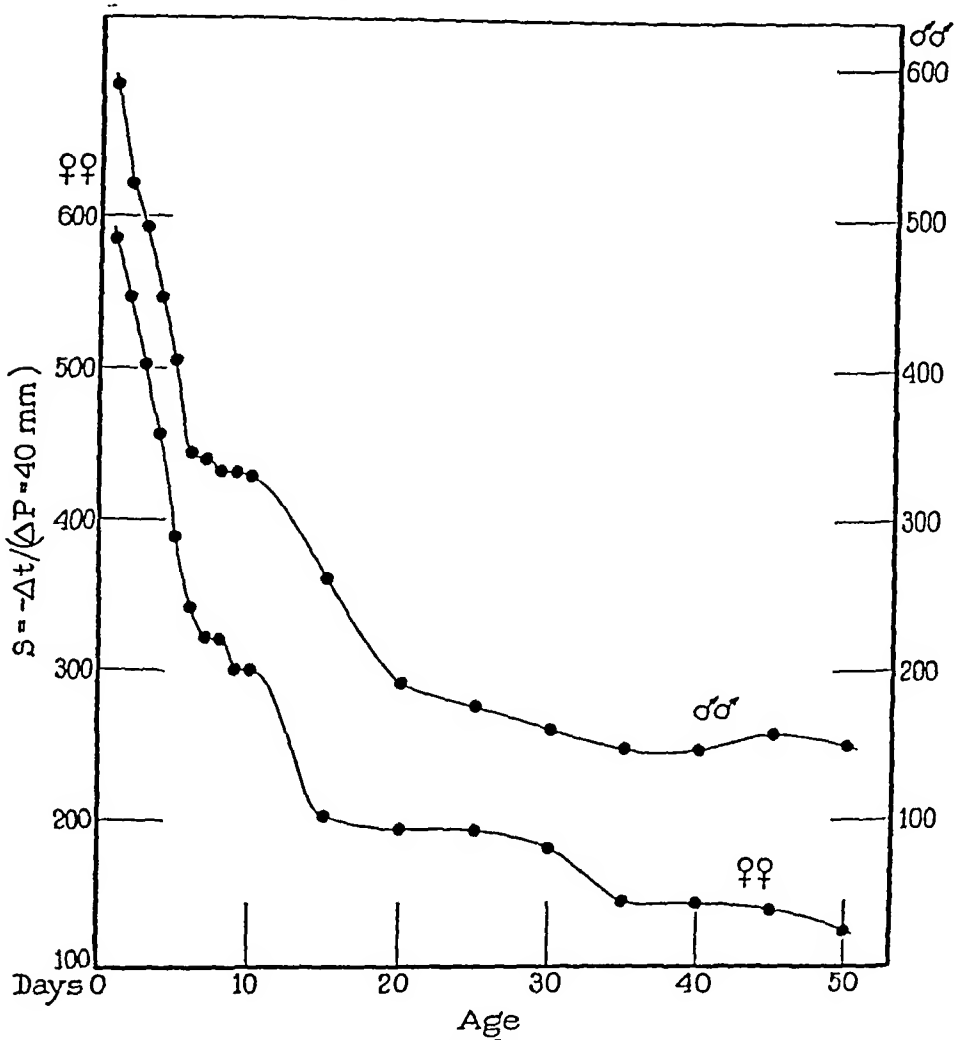
FIG 2 The invasibility of *Drosophila*, stock *F*, for alcohol from its vapor, as a function of age and sex of imago, see text   The invasion coefficient *S* is computed as $-\Delta t/(\Delta P = 40 \text{ mm })$, where *t* is time-to-death (minutes) and *P* is vapor pressure of ethyl alcohol

modification of these periods in other inbred lines and under conditions affecting life duration   Such information would seem essential to the interpretation of the effects encountered under various experi-

mental conditions It is clear that in the data of egg production similar surges of activity are to be detected, and it has been shown
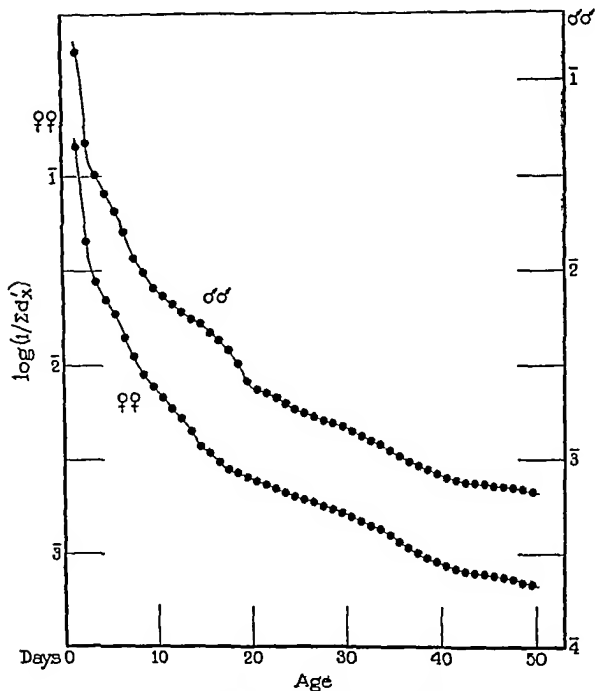
FIG 3 Changes in the rate of accumulation of deaths in male and female *Drosophila* populations, stock *F*, as a function of age At any age $\Sigma d'$ = the total observed deaths up to and including that day The slope of the curve gives the rate of change of $d' /\Sigma d$ Changes in this rate faithfully parallel changes in the rate of alteration of the index of invasibility for alcohol

that cross-over frequencies as a function of age fluctuate in a definitely analogous manner (*cf* Bridges (1927), (1929))

SUMMARY

Periodic accelerations in the decreasing resistance of adult *Drosophila* (pure line) to the penetration of alcohol vapor are specifically paralleled by changes in the rate of accumulation of deaths in a life duration experiment with the same line   The necessary interpretation is that there occur under constant conditions periodic fluctuations in the speed of general metabolic events, marking the limits of what may be termed metabolic instars

CITATIONS

Alpatov, W W , 1932, *J Exp Zool* , **63,** 85
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Crozier, W J , Pincus, G , and Zahl, P A , 1935–36, *J Gen Physiol* , **19,** 523

SUBSTANCES AFFECTING ADULT TISSUE IN VITRO

I. The Stimulating Action of Trypsin on Fresh Adult Tissue

By HENRY S SIMMS AND NETTIE P STILLMAN

(*From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York*)*†

PLATE 1

INTRODUCTION

Previous workers (1–5) have grown various normal adult tissues *in vitro* and have found that these cultures resemble those from embryo tissue, but are harder to maintain

In this and subsequent papers a study of dormant adult tissue will be described   The dormancy is characterized by the *lag period* preceding the onset of growth *in vitro*   Adult chicken aorta has a lag period of 3 to 5 days before the first new fibroblasts can be seen, while embryo tissue starts to grow in a few hours (6)   In this paper it will be shown that digestion of the tissue with trypsin not only shortens this lag period (*i e*, overcomes the dormancy) but also increases the growth rate

EXPERIMENTAL

Our methods differed from the standard tissue culture technique (7–12) in the following details   In each experiment a chicken was bled aseptically from the carotid artery (aided by suction) and was then killed either by clamping the trachea or with ether   Using sterile technique the thoracic aorta was removed,

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and sometimes other tissues These tissues were placed temporarily in "glucosal" solution (containing neither NaHCO₃ nor phenol red) until the aorta had been stripped of its adventitia and examined for fatty areas The tissue could be stored 3 or 4 days, if desired, in the ice box in pH 7 4 Tyrode solution (containing 0 7 gm of NaHCO₃ and 0 05 gm of phenol red per liter) providing the aorta had not been split (see below) but it gave best results when fresh

Treatment of Tissues with Trypsin —The portion of the thoracic aorta which was to be used on a given day was split through the media The outer media was discarded The piece containing the inner media and intima was used It was cut into strips about 1 5 × 2 0 mm Four of these strips were used for each part of an experiment and were placed in a 13 × 100 mm stoppered tube with 1 5 ml (or 2 0 ml) of sterile trypsin solution The pH was adjusted to 7 6 as indicated by phenol red in the solution This adjustment was made with sterile CO_2-air mixtures (about 3 per cent) with the addition, if necessary, of a little 0 15 N NaOH (or NaHCO₃) during the digestion Usually 0 1 per cent Fairchild's trypsin was allowed to act 3 hours at 37°C or, better, 24 hours at 22°C The experiments were controlled by tissue treated with Tyrode solution under the same conditions As a further control fresh tissue was planted without treatment

Planting the Tissue —After this treatment the four strips of tissue from each tube were cut into pieces 0 6 mm in diameter and were planted in two special 32 mm Carrel flasks, 16 pieces in a flask Before the tissue was introduced about 0 08 ml of chicken plasma (which had been diluted with two parts of Tyrode solution, containing phenol red) was spread over the bottom of each flask The sixteen pieces of tissue were then planted in even rows with the aid of a metal guide The flasks were corked and allowed to stand ½ to 1 hour until the plasma clotted The surface was then rinsed with Tyrode solution which was drawn off by gentle suction 0 7 ml of diluted plasma was added, spread over the surface, and allowed to clot The pH was adjusted to about 7 3 with 5 per cent CO_2-air mixture introduced with a sterile gas injector (see Text-fig 1) The flasks were placed at 37°C Fresh CO_2-air was added each day

Evaluation of Growth [1] Each colony was examined daily under the microscope The nature of the growth was such that the following system of evaluation was found most satisfactory (see Text-fig 2)

        0 = no growth
    1 — — (= 0 25) = 1 to 6 new cells
      1 — (= 0 5) = about 10 new cells
        1 = about 30 new cells
        2 = growth extended into medium (about 90 new cells)
        3 = abundant growth but not visible to naked eye
        4 = new growth clearly visible to naked eye
        5 = colony 1 cm or more in diameter

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[1] The word "growth" is used to indicate the production of new cells visible under the microscope
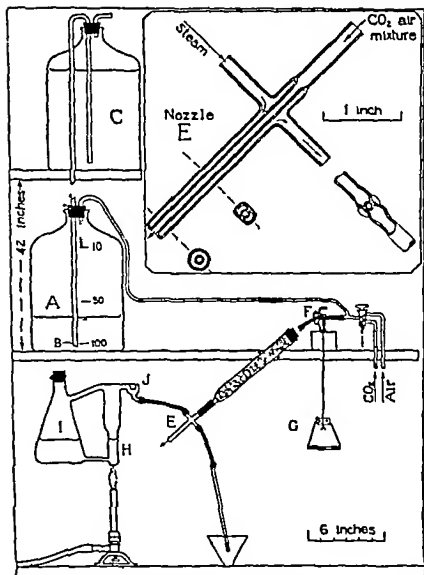
TEXT FIG 1 A sterile gas injector for introducing CO$_2$-air mixtures into flasks or tubes Steam from boiler tube H, keeps nozzle E, sterile The gas mixture from bottle, A, is sterilized by passing through sterile cotton, D, and is injected into the culture flask through E The stopper of the flask can be placed in the sterile cup, J
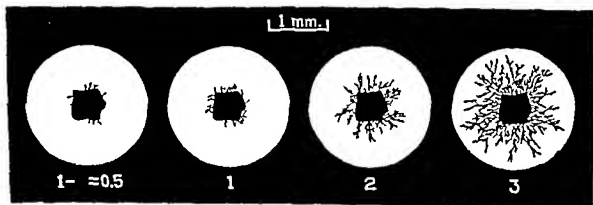
TEXT FIG 2 Amount of growth corresponding to the indicated ratings

Intermediate ratings of 1+, 2+, 3+, and 4+ (equalling 1 5, 2 5, 3 5, and 4 5 respectively) were also used

The sum of the ratings of the sixteen colonies in a flask gave the "growth value" of that flask   Each value in this paper is the mean of two duplicate flasks (thus representing 32 pieces of tissue)

Enzyme Solutions

The trypsin solutions were prepared as described below and were then sterilized by Berkefeld filtration and stored in the refrigerator

Fairchild's trypsin was made up as a 1 per cent suspension, filtered through paper, then through a Berkefeld filter, and kept as a stock solution   This "1 per cent" solution was diluted with 9 volumes of Tyrode solution before using

A "purified trypsin" was prepared from Fairchild's trypsin by fractionation with ammonium sulfate   It was dialyzed against water, then against Tyrode, and used without further dilution

Northrop's *crystalline trypsin* and *chymotrypsin*[2] were made up to 1 per cent solutions in 0 003 M HCl and dialyzed against water, against saturated sodium chloride, then against water, and finally against 0 003 M HCl   They were diluted with Tyrode before use

Papain solution was prepared as a 1 5 per cent solution   This was activated by adding one-fifth volume of a neutralized 1 per cent solution of cysteine hydrochloride and heating 1 hour at 36°C   In using this on a tissue a sufficiently low pH was obtained by introducing a 40 per cent CO_2-air mixture   This did not materially injure the tissue

Activity of Trypsin—The proteolytic activities of the trypsin solutions were determined by digestion of casein solution using the procedure of Northrop and Kunitz (13)

5 0 ml of 5 per cent casein solution was warmed to 35 5°C   1 0 ml of enzyme solution (or 0 5 ml diluted to 1 0 ml) was added   The mixture was maintained at 35 5°C for 20 minutes   Trichloracetic acid was then added and the non-protein nitrogen was determined   The weight of non-protein nitrogen liberated per milliliter of the enzyme solution was taken as the measure of activity

Treatment of Cultures with Trypsin—To each flask culture 1 ml, or less, of 0 1 per cent Fairchild's trypsin solution (in Tyrode) was added   The flasks were placed at 37°C and observed at frequent intervals   As soon as the digestion in a given flask had reached the cells the fluid was quickly drawn off and fresh plasma was added   Sometimes when the digestion was slow, the fluid was replaced with serum at the end of 4 hours, and the treatment repeated the next day   Caution was then necessary to prevent complete liquefaction of the clot

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[2] These crystalline enzymes were obtained through the kindness of Dr John H Northrop

Stimulation of Fresh Tissues by Trypsin

There is a lag period of 3 to 5 days before the first growth of fibro
blasts can be seen from adult chicken *thoracic aorta* which has been
planted in a medium of diluted plasma and kept at 37°C   But if the

TABLE I

Stimulation of Abdominal Aorta with Trypsin

The digestion was followed by 2 days at 4 C in Tyrode solution (Exp 61C)

Treatment before planting	Growth values after planting					Relative growth on 5th day
	1 day	2 days	3 days	4 days	5 days	
Tyrode control	0	0	0 5	2 0	3 0	1
Trypsin 3 hrs 37 C	0	0 7	2 0	5 7	8 5	3

TABLE II

Stimulation of Adult Chicken Liver   (Exp 30A and B)

Tissue treatment before planting	Growth values after planting				
	1 day	2 days	3 days	4 days	5 days
Untreated	0	1 2	5	8	11
Trypsin 3 hrs 24 C	0	3 0	8	10	19

TABLE III

Dog Granulation Tissue, Stimulated by Trypsin

(2 hours at 37°C and 22 hours at 0), planted in chicken plasma (Exp 49)

Tissue	Treatment before planting	Growth values after planting		
		1 day	3 days	5 days
Vascular area (newer growth)	Tyrode control	0	0	0
	Trypsin treated	2 5	6	9
Fibrous area (older growth)	Tyrode control	0	0	0
	Trypsin treated	7	12	19

tissue is first treated with trypsin its lag period is definitely reduced
Furthermore (see Text figs 5, 7, 8, 9, and 10, discussed below) the
growth rate is greatly accelerated
   Other adult tissues are similarly stimulated   These include the
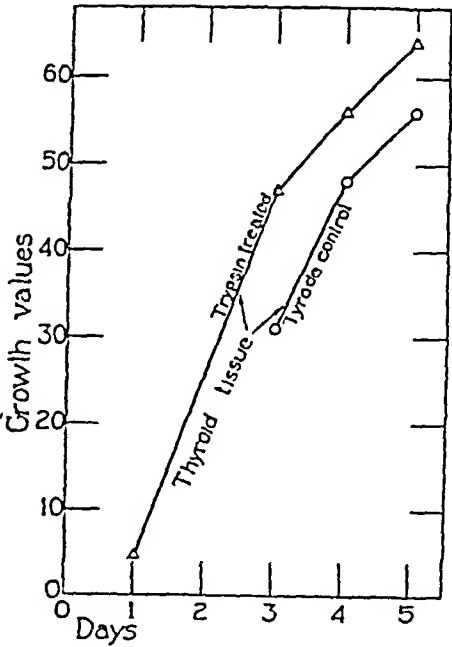
Fig 3
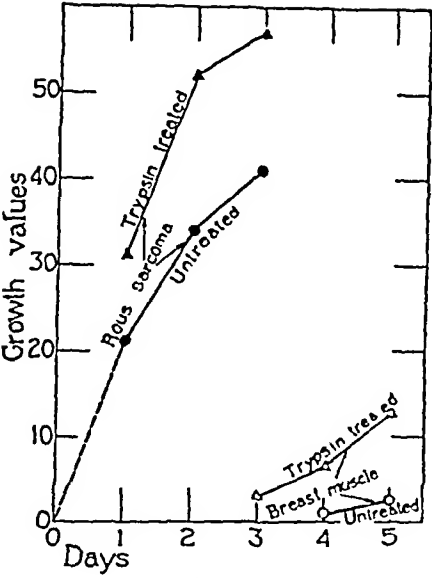
Fig 4
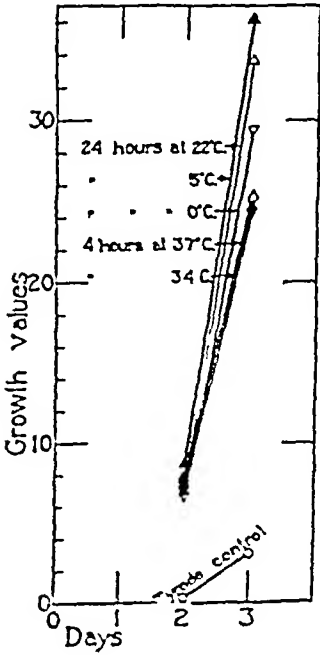
Fig 5
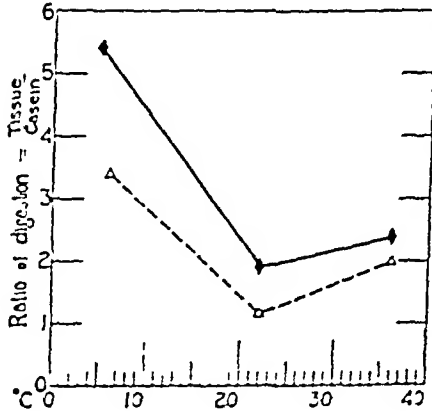
Fig 6

abdominal aorta (Table I), adult *liver* fibroblasts and epithelial cells (Table II), adult *thyroid* (Text fig 3), muscle fibroblasts (Text fig 4), and dog *granulation tissue*[3] (Table III)

Rous sarcoma tissue was stimulated by trypsin treatment (Text-fig 4) even though the untreated tissue had no lag period Of a number of human brain tumors[4] which were studied one *meningioma* grew 15 per cent faster after 2 5 hours in trypsin at 37°C , and one glioma was slightly stimulated (not evaluated)

The stimulation of initial growth of dormant tissue seemed to depend upon obtaining just sufficient digestion of the tissue Too much digestion killed the tissue At the optimum point the tissue was perceptibly softer (but we do not believe this softness caused the stimulation) and was more translucent as seen under the microscope It sometimes contained visible round cells which later developed into fibroblasts [5] The digested aorta tissue, when stained with hema toxylin, was seen to have lost its mucoid material The digestion removed 10 to 20 per cent of the nitrogen from the tissue with a corresponding increase in the nitrogen of the digestion fluid There was considerable variability between chickens in the susceptibility of their tissues to digestion and this did not seem to be related to age, sex, or breed

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TEXT FIG 3 Stimulation of thyroid tissue by trypsin (Exp 65A)

TEXT FIG 4 Stimulation of Rous sarcoma, and of breast muscle fibroblasts, by trypsin digestion (2 hours at 37°C) (Exp 48)

TEXT FIG 5 Relative growth after trypsin treatment under different condi tions (Exp 63B)

TEXT FIG 6 Relative digestion of aorta tissue and of casein by trypsin at different temperatures The solid line represents an experiment (65D) in which the digestion fluids were maintained at pH 7 6 The duration of the digestions were 24 hours at 5°C and at 22 C , and 4 hours at 36°C The broken line repre sents a similar experiment (64A) in which the pH of the tissue digestion fluids dropped to lower values

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[3] Kindly furnished us by Dr Margaret Murray It was prepared by planting small tubes in dog muscle for 12 days

[4] Surgical material obtained with cooperation of Dr Abner Wolf

[5] Rous and Jones (14) obtained similar round cells by drastic digestion of em bryo cultures We likewise observe a retraction of fibroblast processes during partial digestion of our cultures (as in Fig 1, Plate 1)

The control tissues in Text-figs 3, 5, 7, 8, 9, 10, and 11 were treated with Tyrode solution under the same conditions as the trypsin treatment   Most of the experiments were also controlled with fresh untreated tissue (as in Text-fig 4)   There was no significant difference between the Tyrode controls and the untreated controls

Temperature of Treatment —Usually a slow action at a low temperature stimulated better than the faster digestion at higher temperature In Text-fig 5 the relative stimulations under different conditions are compared

The degree of stimulation at 0°C was somewhat surprising   It seemed strange that sufficient digestion could occur at that temperature   This is explained by the results in Text-fig 6 in which the amounts of nitrogen liberated from aorta tissue and from casein are compared under different conditions   If the relative susceptibility
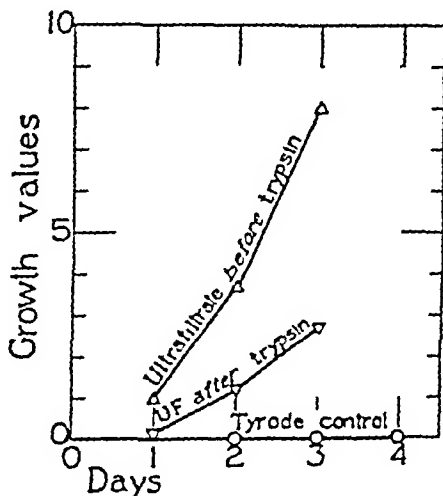
TEXT-FIG 7   Stimulation produced by 3 days incubation of the tissue in serum ultrafiltrate *before* digestion with trypsin (Exp 55C)

(to tryptic digestion) of aorta and of casein were independent of temperature then the three points of each experiment would lie in a horizontal line   (The height of the line is not important)   If we take 22°C as a standard then the experimental results show that the susceptibility to digestion was relatively greater for aorta than for casein at 36°C and considerably greater at 5°C   In other words,

aorta was more readily digested at refrigerator temperatures than would be expected from observations on casein

Incubation of tissue in serum ultrafiltrate (or serum) *before* trypsin digestion produced a much better stimulation than the same treatment after the digestion (Text fig 7) The serum ultrafiltrate contains a stimulant (the 'A factor', see later paper (17)), needed for the initial growth of adult tissue The A factor may render the cells more susceptible to the stimulating action of trypsin The presence of this agent *during* the digestion increased the stimulation only 10 per cent However, the presence of serum in the trypsin solution prevented digestion and gave a stimulation only equal to that of serum alone

Stimulation of Tissue Cultures by Trypsin

Flask cultures of adult chicken aorta fibroblasts which have reached a state of partial degeneration and retarded growth have been treated with trypsin until the plasma clot has been digested sufficiently to expose some of the cells Fresh plasma was added and allowed to clot There was an immediate vigorous stimulation to renewed growth (while control cultures failed to grow appreciably whether they were washed with heparin plasma, serum, serum ultrafiltrate, Tyrode solution, or embryo extract, or if untreated) Plate 1 illustrates one of many such cultures This untreated 11 day culture had not grown for 3 days and was partly degenerated when treated with trypsin Fig 1 was taken immediately after digestion, Fig 2 after 20 hours, and Fig 3 after 3 days

Similar stimulation has been obtained with cultures of Rous sarcoma, Walker rat sarcoma 319 cultures,[6] and a human meningioma culture

Weekly digestion with trypsin (and intermediate washings with serum or serum ultrafiltrate) has made it possible to maintain adult cultures in the same flasks for long periods, but this is not recommended owing to the difficulty of controlling the digestion

The gentle digestion, by leaving the colonies more or less intact, has made it possible to study the stimulating mechanism (see below)

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[6] These pure sarcoma cultures had been grown *in vitro* over a year and a half by Dr Warren H Lewis who furnished them to Dr Joseph Victor, of this department

It has no similarity to the drastic digestion of embryo cultures by Rous and Jones (14) and by Rous, McMaster, and Hudack (15)

Mechanism of the Trypsin Stimulation of Fresh Tissues

Considering first the action of trypsin on fresh tissues (rather than on cultures) three explanations suggest themselves [7] First, the stimulation might be due to protein split products which Baker and Carrel (16) showed would stimulate active cultures of embryo fibroblasts   Second, the stimulation might be due to lipase or some other impurity in the Fairchild's trypsin, and not due to its proteolytic action   Third, the trypsin might stimulate solely because of its proteolytic action on the tissue

Protein Digestion Products —Our use of trypsin was originally intended to stimulate growth by virtue of the protein split products produced by the digestion (16)   However, we obtained results which could not be explained on this basis   In several experiments tissues were thoroughly washed *after* trypsin treatment, previous to planting

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TEXT-FIG 8 Comparison between the growth stimulation of tissue washed before, and of tissue washed after trypsin treatment (Exp 51A)   The tissues were washed 1 day in Tyrode solution at 0°C

TEXT-FIG 9 Stimulation produced by three trypsin solutions of different purity but equal activity toward casein (Exp 27A)

TEXT-FIG 10 Stimulation produced by equally active solutions of Northrop's crystalline trypsin and chymo-trypsin compared with commercial trypsin (Exp 59B)

TEXT-FIG 11 Growth stimulation produced by papain compared with trypsin (Exp 60C)

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[7] It might be thought that the softening of the tissue is mechanically responsible for the stimulation of growth   We do not believe this plays an important rôle   Hardness cannot account for the dormancy of adult tissues, hence softness cannot account for the onset of growth   Soft tissues such as liver or brain have as long a lag period as the firm aorta tissue   It may be seen in Table III that none of the dog granulation tissue grew without the action of trypsin, in which case the firmer tissue grew faster

Furthermore, the dormant cells in an old culture flask are surrounded, before digestion, with a medium much softer than the aorta tissue after digestion What is more growth is generally better in firm plasma clots than in soft ones
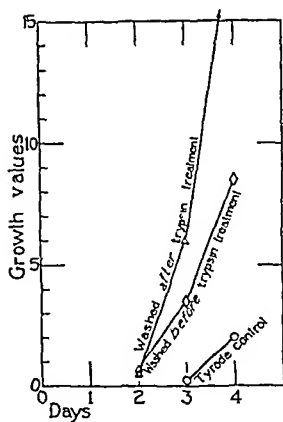
Fig 8
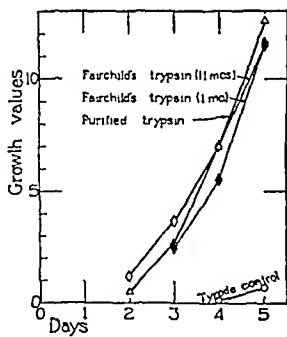
Fig 9
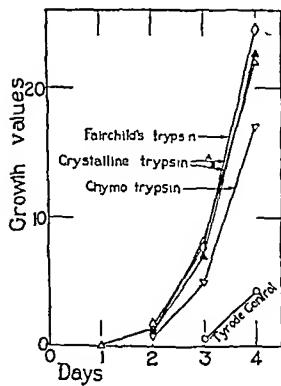
Fig 10
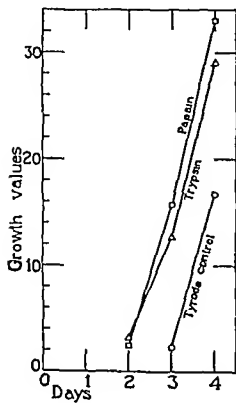
Fig 11

These tissues grew as well as, and sometimes better than, the unwashed ones   Fig 8 gives an example [8]   Note the growth on the 4th day   It is seen that both of the trypsin treated tissues were markedly stimulated (as compared with the control) but the tissue which was washed *after* the digestion grew much faster in spite of the

TABLE IV

Determination of Trypsin Activities

5 0 ml of 5 per cent casein digested with enzyme solution for 20 minutes at 35 5°C (Exp 27A)

Trypsin solution	Volume of enzyme solution	Concentration of enzyme	Non protein nitrogen liberated	Relative activity	Calculated concentration for equal activity
	ml	*per cent*	*mg per ml of enzyme*		*per cent*
Fairchild's (11 mos)	1 0	0 1	0 49	100	0 100
Fairchild's (1 mo)	0 50	0 1	2 2	453	0 022
Purified	0 50	X*	0 87	174	0 58X

* X equals the unknown concentration of trypsin in the purified solution

TABLE V

Determination of Trypsin Activities

5 0 ml of 5 per cent casein digested with 1 ml of 0 2 per cent enzyme solution for 20 minutes at 35 5°C (Exp 59B)

Trypsin solution	Non protein nitrogen liberated	Relative activity	Calculated concentration for equal activity
	mg per ml of enzyme		*per cent*
Fairchild's	3 0	00	0 100
Crystalline trypsin A	6 0	200	0 050
Crystalline trypsin B	5 4	180	0 055
Crystalline chymo trypsin	6 6	220	0 045

removal of digestion products   Had the stimulation been due to the digestion products the reverse effect would have been observed

It might be supposed that some of the digested proteins remained

[8] Text-fig 8 should not be confused with Text fig 7   In one case the tissue was ashed in Tyrode solution at 0°C   In the other case the tissue was incubated at 37°C in the presence of the stimulating " A factor "

in the tissue despite the washing   However, we have found that
the digestion fluid does not stimulate, but on the contrary is slightly
inhibitory (see the following paper)   The possibility of stimulation
due to trypsin retained by the tissue is remote, since trypsin incor
porated in the clot of a culture does not stimulate, and furthermore
tissue which has been insufficiently digested has a good opportunity
to adsorb enzyme—but is not stimulated   Hence we may discard
the idea that the trypsin stimulated the initial growth of adult tissue
because of protein split products [9]

Impurity Versus Proteolytic Action —The suggestion that the crude
trypsin, which we first used, contained an impurity (such as lipase[10]
or another enzyme, or some non-enzymatic substance) which pro
duced the stimulation, is untenable in view of the results obtained
by comparing enzymes of different degrees of purity   In Table IV
are the relative activities of three trypsin solutions, as tested on
casein   The values in the last column indicate what concentrations
of the three enzymes should have equal proteolytic activity [11]

The three enzyme solutions were then diluted to these (equally
active) concentrations and tested on adult aorta tissue   The subse-
quent growth of these tissues, as indicated in Fig 9, shows that the
three enzyme solutions produced about equal stimulation   The first
trypsin solution had been kept 11 months and contained 4 5 times as
much solid matter per unit of activity as did the second solution
The third was a purified trypsin   Hence the amounts of impurities
were quite different in the three solutions, but since the activities
were about equal, and the stimulations were practically equal, it
would appear that the stimulation depends upon proteolytic activity

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[9] We have found (in unpublished experiments) that protein digestion products
prepared by the methods of Baker and Carrel (16) do produce a mild stimulation
of the initial growth of adult aorta tissue under favorable conditions   However,
under the conditions of the experiments reported in this paper, these products
appear to play a negligible rôle in the stimulation of digested *tissue*   On the other
hand there is evidence that digestion products do play a small, but minor rôle
in the stimulation of digested *cultures* (see text)

[10] Evidence is also given in a later paper (17) that the stimulating effect of
serum is not due to the lipase contained in it

[11] This calculation assumes that the activity was proportional to the concen
tration of enzyme   This was roughly true in this range of concentrations

Similar results were obtained in another experiment in which Northrop's crystalline trypsin and some of his crystalline chymotrypsin were compared with commercial trypsin   In Table V the activities of these solutions toward casein were compared   The concentrations for equal proteolytic activity are given in the last column [11]   These concentrations of the enzymes when used on aorta tissue produced about equal stimulation as seen in Fig 10   The two solutions of Northrop's crystalline trypsin were prepared separately from the same solid material

Papain —In Fig 11 it will be seen that papain stimulates the growth of adult tissue in the same manner that trypsin does   Hence the stimulation seems to be purely one of proteolysis   Owing to the necessity of treating the tissue at a lower pH with papain than with trypsin it was not attempted to compare their activities quantitatively

Inhibitor —If we accept the above data as indicating that these enzymes stimulate because of their proteolytic activity we are confronted with a new question, namely, why the digestion of tissue protein should stimulate growth   We suggest that the enzymes digest away an inhibitory protein material, and thus remove it from the environment of the cells   The following paper will show that the digestion fluid contains an inhibitor

Mechanism of Trypsin Stimulation of Tissue Cultures

Returning now to the stimulation of tissue *cultures* (see Plate 1) several explanations suggest themselves   It might be supposed that the stimulation resulted from the products of protein digestion (16)   However, washing away the digestion fluid before adding the new clot resulted in only a slight decrease in the stimulation   This indicated that the digested proteins did aid the growth slightly but that they played only a minor rôle [9]   Furthermore, the addition of digested plasma[12] to cultures in an advanced state of growth failed

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[12] This digested plasma was obtained by digesting plasma clots in Carrel flasks under conditions identical with digestion of the plasma medium of cultures (except that no tissue was present)   The enzyme in this digest was not heat killed but control experiments showed that the enzyme could not have been toxic to these cells

to produce any conspicuous stimulation (although similar cultures were definitely stimulated by digestion to immediate and prolific growth)

These conclusions are further substantiated by many observations on partly digested cultures Within a given culture marked stimulation was obtained only in those portions where the digestion had removed the clot in close proximity to the cells Had the digestion products been the sole cause of this stimulation then these products should have also diffused through the slightly thicker clot in other parts of the same culture—but in reality these other portions were only slightly stimulated, if at all

It might also be supposed that renewal of the plasma clot added some needed material, but washing the cultures with serum (or heparin plasma) failed to stimulate in the way that trypsin did Hence if serum (or plasma) contains such a needed material it must be too slowly diffusible to pass through the medium The only poorly diffusible stimulant in plasma that we know of is globulin (17), and this is only mildly active, especially in the presence of albumin Renewal of the fibrin cannot be essential since the new growth can extend into unused portions of the old clot to some extent

We have found only one explanation which fits our observations, namely that the old clot immediately surrounding each cell contained inhibitory material which was too poorly diffusible to be washed away The trypsin in digesting the clot dissolved the inhibitor with it

It seems unlikely that such an inhibitor could have been produced by the plasma Aging of plasma (in the ice box) for a year does not materially affect its ability to support growth (unpublished data) and plasma that had been incubated several days before use was still stimulating Hence this inhibitor, if it exists, is a product of the cells

SUMMARY

Adult tissue is characterized by a lag period of several days preceding the onset of growth *in vitro*

Treatment of fresh adult tissues with trypsin before planting them in culture flasks stimulated the tissues to grow sooner and more rapidly

Best stimulation was obtained by slow digestion at low temperature The tissues lost nitrogen during the digestion Lowering the temperature from 22°C to 5°C reduced the digestion of aorta tissue much less than it reduced the digestion of casein

Washing the tissue after trypsin treatment resulted in better stimulation

Trypsin solutions of different degrees of purity, when diluted to equal activity toward casein, gave equal stimulation to the tissue growth These included solutions of Northrop's crystalline trypsin and chymo-trypsin

Papain also stimulated growth in a similar manner

The results indicate that this stimulation of tissue growth is due entirely to proteolytic action

Cultures of adult fibroblasts (and some tumor cultures) having reached a state of retarded growth have been treated with trypsin to digest away most of the plasma clot (used as a medium) Fresh plasma has been added to renew the clot This treatment has resulted in an immediate renewal of growth

Reasons are given for supposing that the cells produce an inhibitor *in vitro* which they deposit in the surrounding clot, and which is removed by the action of trypsin

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EXPLANATION OF PLATE 1

FIG 1 An 11 day culture of adult chicken aorta fibroblasts after digestion with trypsin The dark area is part of the original tissue (culture colony 2779 14, F7 11), ×180

FIG 2 The same field after 20 hours (F7 18), ×180

FIG 3 The same field after 3 days (F9-8), ×180

SUBSTANCES AFFECTING ADULT TISSUE IN VITRO

II A Growth Inhibitor in Adult Tissue

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INTRODUCTION

In another paper (1) it was shown that the initial growth of adult chicken aorta tissue was greatly accelerated if the tissue had been digested with trypsin (or papain) previous to being planted in a culture medium It was suggested that this stimulation resulted from the removal of an inhibitor from the adult tissue by the action of the enzyme

In the same paper it was shown that dormant tissue cultures (in addition to fresh tissues) could be stimulated to renewed growth by the action of trypsin, and it was suggested that this resulted from the removal of an inhibitor which had been produced by the cells and deposited in the medium

This paper will show that an inhibitor can be separated from the fluid in which adult aorta tissue has been digested

Selection of Tissue

In choosing a suitable adult tissue for studying dormancy, as described in this and other papers, it was necessary to select one of simple structure which gives a clearly distinguishable growth *in vitro*, with a suitable lag period and growth rate and which gives reproducible results Adult heart, liver, thyroid, skin, muscle, and brain were all unsatisfactory Arteries were better but it will be seen in Figs 1 and 2 that these vary considerably The thoracic aorta gave the best results Different layers of the thoracic aorta are seen in Fig 2 to differ in their growth The inner layer of the thoracic aorta has been used in most of our work with satisfactory results This consists of the inner half of the media together with the intima

EXPERIMENTAL

In the previously described stimulation of growth, the volume of dilute trypsin solution was considerably greater than that of the tissue   In attempts to obtain the inhibitor larger amounts of tissue in proportion to the fluid were used

In the first experiments the digestion fluid was tested directly without fractionation   It was prepared by digesting strips of chicken aorta with 0 1 per cent trypsin solution at 20°C for 18 hours under sterile conditions   Part of the fluid thus obtained was tested without heating   Part was heated at 58°C for 20 minutes and part was heated at 100°C for 5 minutes
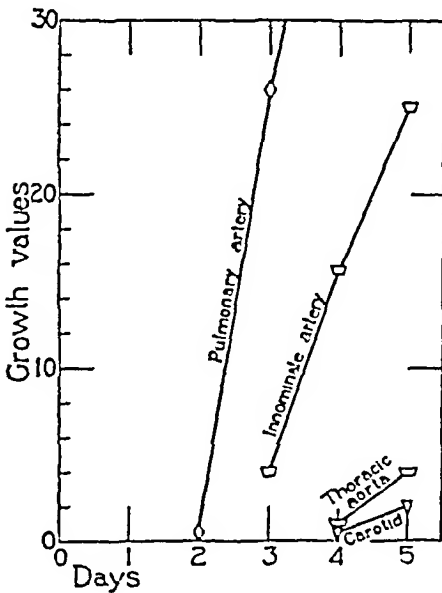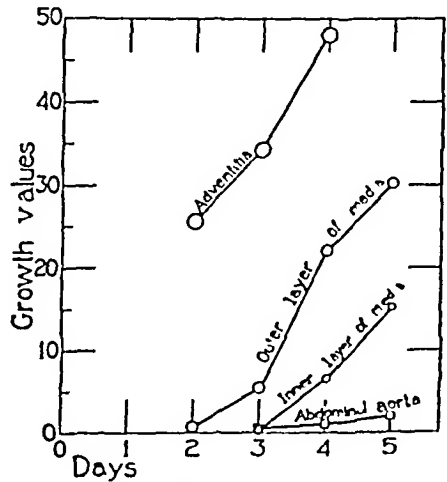
FIG 1   FIG 2

FIG 1 Comparison of growth of various artery tissues (freed from adventitia) from the same adult chicken   The same relation was found in other chickens

FIG 2 Comparison of three layers of the thoracic aorta (also a sample of abdominal aorta)

In later experiments the digestion fluids from dog or sheep[1] aortas were used A typical preparation (I7) was made from 15 gm of sheep aorta (intima and media)   This was minced with scalpels and treated with 20 ml of 0 2 per cent

[1] The latter were obtained from experimental sheep by the courtesy of the Physiology Department

solution of Fairchild's trypsin[2] at 37°C for 3 hours[3]   The pH was kept at about
7 6 by occasional additions of 0 1 ml of 0 5 M NaOH   Near the end of the diges
tion the pH was allowed to drop to 6 8 (a little HCl was added in other experi
ments)   The fluid was then removed by centrifuging and filtering   It contained
0 15 per cent non protein nitrogen and 0 12 per cent protein nitrogen (equivalent
to 0 76 per cent proteins)

This fluid was treated with an equal volume of absolute alcohol and was allowed
to stand at 4°C overnight   The precipitate was dried in a desiccator and half of it
was then taken up in 3 ml of "Na K G" solution[4]   This fraction (I7A1) was
pasteurized at 58 C for 20 minutes and was found to be quite inhibitory (Fig 4)
when mixed with serum (as compared with the serum control and with a portion
of the inhibitor which had been heated at 100°C for 5 minutes)

The above precipitate was produced by the addition of alcohol   The remaining
fluid (45 ml) was next treated with 1 ml of 5 per cent CaCl₂ plus 1 6 ml of 0 1 M
NaOH   The resulting precipitate was allowed to settle at 4 C for 3 hours, was
centrifuged off, and was taken up in Na K G solution   It (I7B) was also found
to be active (Fig 5)

A number of fractions were dialyzed   For example, a CaCl₂ precipitate (I8B,
prepared like I7B, above) was taken up in 5 ml of "Na₀ ₆H₀ ₄Ac" solution[5]   This
was dialyzed 5 hours in a rocking dialyzer (2) against flowing water and 1 hour
against 30 ml of Na K G solution in a swirling shaker, with the gradual
addition of 1 ml of 0 5 M NaOH to the outside solution, to adjust the pH

The method of testing the activity of the inhibitors consisted in incubating
adult aorta tissue in the presence of these materials previous to planting the tissue
in a plasma medium   The inner layer of fresh adult chicken aorta was cut into
pieces 1 5 × 2 mm in size, as previously described (1)   Four pieces were placed
in each of 10 or 11 sterile 13 × 100 mm tubes   To these tubes 1 5 ml portions
of various test fluids, including controls, were added   The inhibitor fractions
were mixed with one third volume of serum   The pH was kept about 7 4 with
CO₂-air mixture for 4 days while the tubes were incubated at 37°C   CO₂ is needed

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by the tissue   The tissue was then cut into 0 6 mm pieces and planted as already described (1)

All test solutions were made up so that they contained glucose and the inorganic constituents of serum   The osmotic pressure was always about isotonic (0 29 osmolar) it having been found that a 40 per cent decrease or increase (to 0 18 or to 0 40 osmolar) could be tolerated but that greater changes are harmful, growth stopping at 0 5 osmolar (70 per cent increase)

DISCUSSION

An inhibitor has been found in the fluid after adult aorta tissue has been digested with trypsin   In Fig 3 the unheated digestion fluid itself (mixed with serum) is seen to inhibit growth of fresh tissue (as compared with the serum control), although it lost this inhibitory power upon heating, and became somewhat stimulating to fresh tissue (perhaps due to the protein digestion products)   The loss of inhibitory power upon heating is attributed to the destruction of an inhibitory material which the tryptic digestion removed from the tissue   That this inhibitor could not be the trypsin itself was shown by experiments in which active trypsin was added in various proportions to serum (to duplicate conditions of testing)   It was never inhibitory

Inhibitory Precipitates —When such a fluid is treated with an equal volume of alcohol it gives a precipitate which is inhibitory (see Fig 4)

The remaining fluid upon the addition of $CaCl_2$, plus a little NaOH, gives a second precipitate which is also inhibitory as can be seen in Fig 5

Since both these precipitates are active and appear to contain the same material, it is not necessary to separate them   The digestion fluid can be treated with alcohol followed by calcium chloride (and NaOH) and the whole precipitate separated   This is active as shown in Fig 6

Dialysis —Several of the preparations were dialyzed   Two of these lost all their activity   Two others seemed to be active but the boiled controls were lost by accident or infection   However, a fraction is seen in Fig 7, to have been inhibitory after dialysis

Sterility and Sensitivity —In some experiments, such as the one illustrated in Fig 3 aseptic technique was followed throughout   The
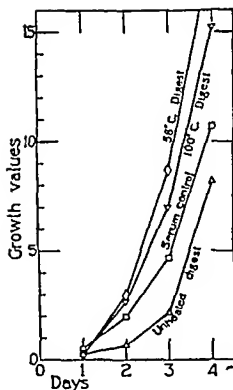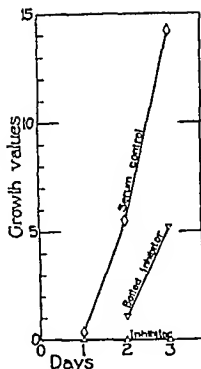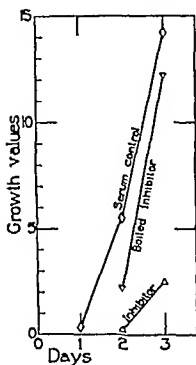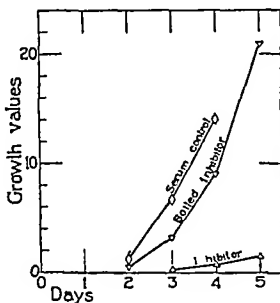
FIG 3 Fluids from tryptic digestion of aorta, mixed with serum and used to treat fresh aorta previous to planting Two portions of the digest were heated as indicated (Exp 34B)

FIG 4 An alcohol precipitate is seen to be completely inhibitory while a portion heated at 100°C permitted growth (Inhibitor 7A1, Exp 50)

FIG 5 A CaCl₂ precipitate (from the fluid after alcohol precipitation) is strongly inhibitory as compared with a portion heated at 100°C , and with a serum control (I7B, Exp 50)

FIG 6 Inhibitory action of a precipitate produced by the addition of both alcohol and CaCl₂ (I4B, Exp 41A)

chicken aorta was sterile at the start   However, in all the experi-
ments using dog or sheep aortas the tissue was not sterile   In these
experiments the material was usually pasteurized at 58°C for 20
minutes at some time during the procedure   The inhibitory fractions
in Figs 4, 5, and 6 were all sterilized in this way

However, this amount of heating can destroy the inhibitory ac-
tivity   It will be seen that the fractions in Figs 3 and 7 which were

tor and lactenin (a substance in milk (3) which produces bacterio stasis) This similarity is confirmed by our observations on the tissue inhibitor which are described in this paper

Both these inhibitors withstand moderate heating at 58°C but are destroyed at 100°C They are both precipitated by the addition of one volume of alcohol, and by addition of the calcium ion Both are dissolved again under conditions favorable for the solution of calcium phosphate They both withstand a moderate digestion with trypsin and are thereby liberated from much of the accompanying proteins Both survive dialysis (i e, they do not pass through the collodion bag) for a limited time providing the action of the trypsin has not gone too far Furthermore, both inhibitors often lose their activity for some unaccountable reason when conditions seem to be carefully controlled

It was therefore decided to test for their identity by seeing if each would perform the function of the other

First it was found that whey (containing lactenin) would inhibit adult tissue growth In Fig 8 it will be seen that pasteurized whey incorporated in the medium in which aorta tissue was planted, caused a reduction of growth as compared with the boiled whey control

However, the reverse test gave a negative result A preparation of tissue inhibitor failed to inhibit the growth of scarlet fever strepto coccus [6] This has not yet been repeated

The Inhibitor in Tissue Cultures —Observations on cultures from adult tissues (1) led us to suppose that the cells produce an inhibitor *in vitro*, which is deposited in the surrounding medium, and which can be removed by digestion with trypsin This inhibitor, if it exists, may be identical with the inhibitor in dormant adult tissue

Tumor Inhibitors —Sittenfield, Johnson, and Jobling (4) found that Rous tumor extract contained an inhibitory protein fraction which could be separated from other proteins and from the causative agent, by precipitation It mitigated the potency of the tumor agents—but not of tumor cells

Murphy and Sturm (5) have worked with an extract from desiccated chicken

[6] This was the same strain of streptococcus which the late Dr Frederic S Jones had used in our previous studies It was found to be still susceptible to lactenin Dr Charles A Slanetz was kind enough to test the action of the tissue inhibitor on this organism

sarcoma tissue which restrained sarcomas in chickens and mice—but not a carcinoma   Furthermore, extracts from desiccated embryo skin placentas and mammary glands inhibited some mouse carcinomas, but not sarcomas   These extracts withstand 55°C but not 65°C, and in this respect resemble both heten r and our adult tissue inhibitor   Whether there is further similarity remains to be determined

Morton and Beers (6) found that an extract of human rectus sheath sometimes restrained rat carcinoma transplants   Unsaturated fatty acids and pancreas extracts have been claimed to retard the growth of chicken sarcomas and rat tumors (7)

Significance —We believe that the tissue inhibitor described in this paper plays a large rôle in restraining growth in the adult animal body, thereby keeping the cells in their normal dormant state   It is suggested that the cells elaborate the inhibitor and deposit it in the surrounding intercellular space where it remains because of it insolubility

SUMMARY

Digestion of adult tissue with trypsin has been shown to stimulate its initial growth *in vitro*   This stimulation appeared to result from the removal of an inhibitory material from the tissue due to the proteolytic action of the trypsin

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SPECTROSCOPY OF CATALASE

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Catalase is a hemin containing enzyme. This fact was rendered very probable by the discovery of a modified hemin spectrum in purified enzyme solutions from animal and plant tissues by Zeile and Hellstrom (1, 2). They found that the ratio between catalytic activity and porphyrin bound iron in a given preparation remained constant in the course of fractionation and inactivation experiments. This ratio may, however, vary considerably in different preparations, even from the same type of tissue. Besides the approximate position of the absorption bands of the enzyme in the visible region, the spectrum of the cyanide and sulfo-complex and that of the pyridine hemochromogen derived from the enzyme hemin were described.

That the compound responsible for the hemin spectrum in catalase preparations is identical with the enzyme, was proven by the observation that it undergoes a cyclic spectroscopic change during the decomposition of ethyl hydrogen peroxide (3). The prosthetic group of the enzyme has been isolated in crystalline form and its identity with protohematin IX has been demonstrated by conversion into dimethyl IX mesoporphyrin ester (4).

The object of the present study was to gain further information concerning the absorption spectrum of the enzyme and to study the effects of certain reagents on this spectrum. It was hoped to arrive at a decision concerning the state of valency of the iron contained in the enzyme.

EXPERIMENTAL

Enzyme Preparations

Purified catalase solutions were prepared from horse liver[1] in the manner described by Zeile and Hellström (1). The activity of the solutions employed in

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this study, as expressed by the monomolecular velocity constant obtained under standard conditions was from $k = 1610$ to 7125

The enzyme solutions are brown in incident light and brown-red in transmitted light   They show the spectrum of the enzyme in layers of 1 to 5 cm , depending on their catalytic activity   In addition to catalase, these solutions contain traces of biliverdin and hepatoflavin   These two pigments do not interfere with observations of the enzyme spectrum in the range of 650 to 400 mμ Furthermore, the solutions contain colorless proteins and electrolytes

Technique

The preliminary experiments were carried out with the aid of pocket spectroscopes (Brown, Zeiss)   The scale of the Brown instrument was calibrated with the aid of a number of emission lines of the mercury arc and of impregnated carbons The Zeiss instrument has a scale permitting direct reading of the wave-length The use of such instruments with small dispersion makes possible the detection of indistinct or faint absorption bands which would be difficult to observe with large instruments   Well defined absorption bands were then measured more accurately with a Hilger wave-length spectrometer.   In case of the comparison of two absorption spectra, they were projected simultaneously into the instruments by means of comparison prisms   The Hilger spectrometer was frequently standardized with the aid of an electric sodium burner (Zeiss) and of the mercury arc   As light sources tungsten filament lamps, ranging in intensity from 60 to 750 c p , were used, depending on the transparency of the objects under study The solutions were contained either in absorption cells of definite layer of thickness, ranging from 0 5 to 3 0 cm , or in a Baly cuvette graduated to a length of 5 0 cm in millimeters   For the experiments dealing with the effect of gases on the enzyme, capillary stopper cells of 2 0 cm thickness of the type described by Warburg *et al* (5) were used   For observations in layers from 10 to 50 cm polarimeter tubes were used

Position of the Absorption Bands of Catalase

The most conspicuous absorption band of catalase solutions, upon direct examination in the visible range, is seen in the red region Besides, two indistinct bands may be seen in the green region   Zeile and Hellstrom (1) have photographed the band in the red, and they have also determined the position of the two bands in the green They give the following positions for these three bands

$$\text{I } 650 \underbrace{646 - 620}_{629} 610, \text{ II } \underbrace{550 - 530}_{540} 520 \underbrace{510 - 490}_{500} m\mu$$

According to these authors, the maximum of extinction in the first band is not situated symmetrically between 646 and 620, but *shifted*

towards the blue range, at about 629 mμ   They did not measure beyond 463 mμ

The absorption band of greatest extinction of hematin protein compounds is situated in the far violet region, near 400 mμ (Soret's band)   In the case of hemochromogens like cytochrome, this band may be directly observed if the red region is shut off by a suitable light filter (6)   Inasmuch as the observation of a strong absorption band of catalase in this region would constitute additional proof for its hemin nature, the present author has attempted to locate this band   Direct observations in the manner described by Warburg were not successful, principally because of the great decrease of luminous intensity of the tungsten filament lamps in this range and because the spectrum of the carbon arc appears no longer continuous in this range   But when various catalase solutions were analyzed by the recording, photoelectric spectrophotometer of Hardy at the Massachusetts Institute of Technology,[2] the Soret band of the enzyme was clearly recorded in diluted solutions   The peak of this band is at 409 mμ, as compared with 436 mμ for the oxygen transferring enzyme No 1 of Warburg and Negelein (7)   From these records the position of the maxima of the three bands in the visible region is taken as follows

$$\text{I} \underbrace{640 - 600}_{622} \quad \text{II} \underbrace{550 - 530}_{540} \quad \text{III} \underbrace{515 - 490}_{505} \text{ mμ}$$

Though there is general agreement with the values given by Zeile and Hellstrom, slight differences exist, as shown above

Effect of pH on the Enzyme Spectrum

The appearance of the enzyme spectrum is the same in the pH range from about 5 to 10   Below and above this hydrogen ion concentration, the spectrum fades and the enzyme is destroyed   The first step in this irreversible process appears to be the detachment of the prosthetic group of the enzyme from its protein carrier   In

acid solution, the protein is precipitated, in alkaline solution it is denatured   By the latter process a new compound, exhibiting a characteristic spectrum, is formed   It shows two absorption bands in the green, one at approximately 575 and the other at 545 mμ The conversion of the enzyme into this compound is slow in 0 05 N NaOH, being complete after 1 hour   In 0 125 N NaOH the transformation takes place instantaneously

Zeile and Hellstrom found a similarity between the spectrum of catalase and that of alkaline hematin   It appears to the writer that a perhaps still closer resemblance exists between the spectra of catalase and methemoglobin   Whereas the band of alkaline hematin in the red is at 616 mμ compared with 622 mμ for catalase, the corresponding band of methemoglobin is centered at 630 mμ (8, 9) Upon visual observation this band has been found between 630 and 620 mμ (10)   The similarity finds its explanation in the fact that catalase and methemoglobin have an identical prosthetic group, parahematin, and differ only in respect to their protein carrier (4) It is desirable to obtain reliable criteria for distinguishing these two substances in solution   A number of such criteria have been found in the course of this work   One of them is the fact that the spectrum of the enzyme is the same between pH 5 and 10   The spectrum of methemoglobin is known to change from the neutral or acid type to the alkaline type at pH 8 5 to 9   Whereas the main bands of neutral or acid hemoglobin in the visible are at 630 and 500 mμ, the bands of alkaline methemoglobin are at 589–579 and 558–535 mμ, with a faint band near 600 mμ (8, 10)   The Soret band of methemoglobin is at 415 mμ

Experiments on the Valency of the Enzyme Iron

The remarkable stability of the catalase spectrum against hydrosulfite and ferricyanide, which are known to react with all other porphyrin iron complexes yet studied,[3] prevented Zeile and Hellstrom (1) from arriving at a definite conclusion concerning the state

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[3] The only other exception known to the writer is the modified cytochrome—a component of certain bacteria   According to Fujita and Kodama (11), the band of this hemochromogen in the red region may be abolished by ammonium sulfide

of valency of the iron in catalase   In preliminary experiments this observation could be confirmed   With ferricyanide, no change in the enzyme spectrum could be detected even after standing for 30 minutes at room temperature   In order to obtain more conclusive evidence the effect of a variety of reducing agents and of compounds, forming stable complexes with either ferric or ferrous iron, on the enzyme was studied

Effect of Reducing Agents on Catalase

Stokes' reagent, alkaline cysteine hydrochloride solution, and hydrogen, activated by colloidal palladium, did not produce any change in the enzyme spectrum   These agents reduce methemoglobin rapidly to hemoglobin

Freshly prepared ammonium sulfide caused the appearance of a new band at 560–590 mμ The band in the red increased in intensity and showed a shift to 640 mμ The solution became black green This phenomenon is probably due not to a reduction of the enzyme but to the formation of a compound corresponding to sulfo-catalase, which was produced by Zeile and Hellstrom by adding hydrogen sulfide

Interesting observations were made when hydrazine hydrate was tried   The addition of comparatively large quantities of 50 per cent hydrazine hydrate to a neutral catalase solution of $k = 2092$ caused a decrease in the intensity of the enzyme band in the red and the appearance of a new band in the yellow   The final spectrum after 30 minutes was

$$\text{I} \underbrace{640-600}_{622} \quad \text{II} \underbrace{598-578}_{588} \quad \text{III} \underbrace{550-540}_{545} \text{ m}\mu$$

The order of intensity of these bands was III, II, I   When this solution was saturated with carbon monoxide, the solution appeared

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or hydrazine hydrate but not by hydrosulfite   It is possible that the band, which these authors describe, is the α band of the oxidized form of the respiratory enzyme which according to Warburg (12), is directly visible in microorganisms of high respiratory activity

cherry red in layers of 5 cm   The spectroscope revealed the presence of two absorption bands

$$I \underbrace{586 - 570}_{578}, \quad II \underbrace{554 - 531}_{542} m\mu$$

Band I was stronger than band II

At first the writer was inclined to interpret these observations as follows   The enzyme is reduced to the ferrous form by the hydrazine hydrate   The ferrous form in turn combines with carbon monoxide   Control experiments, however, which were partly suggested by Professor D Keilin, Cambridge, to whom the author is indebted for his valuable advice, showed that such an interpretation cannot be correct   If the compound formed by the action of hydrazine hydrate were the ferrous form of the enzyme, it should be possible to reoxidize it to the original ferric form of the enzyme Neither treatment with air nor with ferricyanide effected such a reoxidation   Ferricyanide caused a fading of the absorption bands in the visible region, air was without effect   Furthermore, the hydrazine hydrate solution proved to be so strongly alkaline that the phosphate buffer concentration of the enzyme solution was not able to prevent a shift to the alkaline side   Inasmuch as it was observed that comparatively large amounts of hydrazine hydrate had to be used to produce the new compound, it is to be concluded that the latter is not merely the reduced form of the enzyme but an hemochromogen in which hydrazine acts as the nitrogenous base   Hemochromogens form complexes with carbon monoxide

Photodissociation of Carbon Monoxide Complexes of Catalase Derivatives

The photodissociation of hemochromogen-carbonyl complexes may be directly observed with the aid of a spectroscope and of two light sources   A light source of low intensity is arranged in line with the spectroscope and the absorption cell   Its intensity is sufficient to see the absorption spectrum of the CO-complex but not sufficiently strong to cause photodissociation   A 60 c p tungsten filament lamp (110 volts) is satisfactory for this purpose   The second light source is arranged at a right angle to the optical axis   It must be of high

intensity and its light must be concentrated on the side wall of the absorption cell   A 750 c p projection lamp (110 volts) with concentrated filament was used   A similar arrangement has been used by Warburg in a demonstration at the Conference on Biological Oxidations in Heidelberg in 1932 (13)

In preliminary experiments with CO pyridine hemochromogen from blood hemin it was ascertained that on observation with the weak light source, the absorption spectrum of the undissociated CO complex was visible   When the strong light source was turned on, at first the long wave band split up into two components and then both bands shifted towards the blue   In the stationary state the spectrum of the free pyridine hemochromogen was alone visible The process could be reversed by turning off the strong light source

When the same experiment was tried with the carbon monoxide complex of the hydrazine derivative of catalase, no change in its spectrum could be observed upon illuminating with the strong light source   There was a suggestion that the long wave band gained a little in intensity

If solutions showing the catalase spectrum are treated with hydro sulfite in the presence of NaOH and pyridine, the hematin group of the enzyme is detached from the protein carrier and a pyridine hemo chromogen is formed (1)   This hemochromogen combines reversibly with carbon monoxide   The free hemochromogen and the CO complex show the same absorption spectrum as the corresponding protohem derivatives prepared from blood hemin   To 10 cc of a catalase preparation of $k = 2092$, 2 cc N NaOH, 5 cc pyridine, water to make up a volume of 20 cc , and a small amount of solid sodium hydrosulfite were added   After flushing for 5 minutes with carbon monoxide the solution assumed a pink color   It showed the bands

$$\text{I} \underbrace{563 - 568}_{565} \quad \text{II} \underbrace{523 - 537}_{530} \text{ m}\mu$$

when examined in the light of the 60 c.p lamp in the arrangement described above   When the 750 c p lamp was turned on, photo-dissociation was complete within about 1 second   The maxima of the bands of the free hemochromogen are at 557 and 527 mμ Re-

versal of the process began when the strong lamp was turned off, for completion of the recombination a longer period than for that of dissociation was required (about 3 seconds)   The experiment shows that the pyridine hemochromogen obtained from catalase is subject to photodissociation as is also the corresponding derivative from blood hemin (15)

Effect of Carbon Monoxide on Catalase

If catalase were an hematin compound containing *ferrous* iron, it would be expected to form a complex with carbon monoxide   It should be mentioned, however, that cytochrome-c, which in the reduced form is a true hemochromogen, will combine with CO only in rather alkaline solutions (14)   If, on the other hand, catalase in the normal state were a *ferric* compound, but would undergo reversible reduction in the course of its catalytic action on peroxides, such an intermediary ferrous form might be expected to combine with carbon monoxide and to accumulate in the form of this complex   In both cases CO would inhibit the enzymatic reaction in a manner similar to the inhibition of the respiratory enzyme No 1 (Warburg (15)) Sometime ago the writer observed an inhibition of the catalase of leucocytes by carbon monoxide (16)   Shortly afterward, however, this inhibition was explained in terms of an unspecific damage to the enzyme by treating the solutions with a gas   This was indicated by the observation that even hydrogen when bubbled through enzyme solutions may cause a decrease of the activity (17)   The so called inhibition by CO of peroxidase, which appears to be another enzyme with a hemin group (18), has found a similar explanation (19)   Re cently, however, Califano (20) in a short note has reported that catalase is specifically inhibited by CO and that the inhibition may be relieved by illumination of the reaction system   This situation made a reinvestigation desirable

Effect of CO on the Catalase Spectrum —When a solution of catalase is saturated with carbon monoxide, no change in the enzyme spectrum is detectable   Since in some hemin compounds CO will produce merely a slight change in the position of the absorption bands or even only a change in intensity of one band (21), the spectra of solutions of catalase in air and in carbon monoxide were projected

simultaneously into the spectroscope Both spectra appeared identical in every respect The iron of the enzyme therefore appears not to be in the bivalent state In other experiments, a catalase solution of $k = 1610$ was saturated with carbon monoxide and treated with small amounts of 30 per cent hydrogen peroxide while CO flushed the solution There occurred a violent decomposition of the substrate, the spectrum of the enzyme remained apparently unchanged This experiment makes it improbable that the ferrous form of the enzyme is an intermediate in the catalysis

Effect of CO on the Activity of the Enzyme —The experiments were arranged under conditions favorable for an inhibition of the enzyme reaction by carbon monoxide A low substrate concentration was selected in order to facilitate a combination of the enzyme with CO The experiments were carried out at low temperatures because this increases the stability of hemin complexes with CO The rapid decrease of the dissociation constant of CO hemochromogens and of the CO compound of the respiratory enzyme with decrease in temperature has been demonstrated by Warburg and Negelein (22) and Kubowitz and Haas (23) The enzyme concentration was kept small compared with the carbon monoxide concentration The experiments were performed in an atmosphere of pure CO During the reaction with the substrate the system was flushed with CO in order to remove any oxygen formed which might interfere with the inhibition effect (20)

A chamber was constructed which was similar to the vessels used in potentiometric titrations It was provided with in and outlets for carbon monoxide and nitrogen, with a syphon system permitting the addition of reagents without introducing air and the removal of fluid from the vessel A stirrer with a mercury seal and a microburette completed the equipment The chamber was cooled by running ice water The temperature was kept within 5 to 6 5°C The apparatus was shielded against direct light to prevent the photo dissociation of a carbonyl complex The chamber was filled with 10 cc $\text{m}/15$ phosphate buffer, pH 6 8 35 cc 0 02 N H_2O_2, and 4 cc water While the solution was cooled, it was saturated with pure CO In order to avoid mechanical damage of the enzyme, the rate of gas flow was kept low (7 to 22 cc CO per minute) A catalase

preparation of $k = 2092$ was diluted 1 2000 [4] 1 cc of this solution was added to the substrate-buffer system   After 10 minutes the reaction was stopped by adding 5 cc 33 per cent H_2SO_4 The solution was withdrawn from the chamber and the amount of remaining hydrogen peroxide determined by iodometric titration   The zero value was determined in a similar mixture by adding the sulfuric acid before the enzyme   Control experiments were conducted with air and nitrogen instead of carbon monoxide   A total of 45 experiments were performed   A few results are given in Table I

Little, if any, inhibition by CO was observed in these experiments This inhibition was smaller than that produced by flushing the solution with nitrogen   These results, then, lend no support to Califano's claim that catalase is specifically inhibited by CO   Califano[5] worked under conditions which were less conducive to an inhibition effect than those just described   He used a manometric method   Although he states that oxygen will prevent the inhibition, oxygen accumulated in his vessels during the catalysis, and it was by the amount of evolved oxygen that he measured the progress of the reaction   The temperature ($20°$) and the substrate concentration ($0 17$ N) in his experiments were much higher than in those here reported   He states that the inhibition was around 35 per cent and the reactivation by light around 15 to 20 per cent   At present no satisfactory explanation can be offered for the discrepancy in the results of Califano and of the writer

Effect of Fluoride on the Catalase Spectrum

The experiments with oxidizing and reducing agents or with carbon monoxide allow no final decision as to whether catalase contains ferric or ferrous iron   Fluoride has a marked affinity for ferric but not for ferrous iron   It reacts with catalase to form a stable compound with a characteristic absorption spectrum

The absorption band of the enzyme in the red is centered around 622 mμ When neutral catalase solutions are made weakly acid by

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[4] It is advisable to use ice cold distilled water for the preparation of the diluted enzyme solution   Fresh dilutions must be prepared each day

[5] Professor L Califano was kind enough to supply the writer with information concerning these details of his experiments

adding primary potassium phosphate, the spectrum remains un changed When neutral sodium fluoride is added to the acid solution, the enzyme band in the red splits into two components The new bands are better defined and thinner than the original band

25 cc. of a catalase solution of $k = 2092$ were saturated with solid $KH PO_4$ 1 gm of pure sodium fluoride was added and the solution was filtered The solution appeared greenish in incident and orange

TABLE I

Effect of Carbon Monoxide on the Activity of Catalase

Experimental series No	Atmosphere	Substrate decomposed	Notes
I	Air	1 89 cc. 0 1 N	Air at rest
		1 86	
		1 46	
VI	Air	3 93	Air at rest
	Nitrogen	1 9	Flushed with N_2
	Air	3 93	Air at rest
	CO	3 16	Flushed with CO
VII	Air	3 65	Air at rest
	CO	3 53	Flushed with CO
	Air	3 23	
	CO	3 35	
	CO	3 36	
VIII	Air	3 26	
	CO	2 99	7 cc CO per min
	Air	2 88	
	CO	3 05	22 cc CO per min
X	Air	5 0	
	CO	4 6	26 cc. CO per min
	Air	5 3	
	CO	4 52	
	Air	5 15	

red in transmitted light The long wave bands were measured in 5 cm layer with the Hilger spectrometer

$$\text{I} \underbrace{624 - 617}_{620} \quad \text{II} \underbrace{604 - 597}_{600} \quad 588 \text{ m}\mu$$

Band I is better defined than band II, but the latter is stronger Besides these bands the bands of the enzyme at 540 and 500 $m\mu$ could be seen The pH of the final mixture was 5 88

The complex between catalase and fluoride is stable for several days at room temperature

The reaction of catalase with fluoride is evidence for its constitution as a *ferric* compound   It also affords a simple means of differentiating the enzyme from methemoglobin   The red band of methemoglobin is not split by fluoride but merely shifted from 630 to 610 mμ (24, 25)   The exact position of the band of fluormethemoglobin depends on the pH (26)

Effect of Nitric Oxide on the Catalase Spectrum

Catalase reacts with NO in neutral solution   The color of the enzyme solution changes from green-brown to red   The NO compound has a characteristic spectrum with two bands in the visible range

Catalase solution (k = 2092) was placed in a capillary stopper cell   The air was displaced by pure nitrogen   Nitric oxide was prepared in the apparatus described by Warburg (27)   The enzyme solution was saturated with NO and protected against oxygen by the capillary stopper   The measurement with the Hilger spectrometer had the following result

$$\text{I} \underbrace{581 - 572}_{576}, \quad \text{II} \underbrace{549 - 534}_{541} \text{ m}\mu$$

Band I is much stronger and better defined than Band II   The short wave edge of II was difficult to ascertain   There is a shadow at 610 mμ, but this is only visible with a pocket spectroscope

The NO-catalase compound has a higher extinction than the free enzyme   3 to 4 cm layers of the enzyme solution are required to clearly see the absorption band in the red but the bands of the nitric oxide complex are already distinct in 0 5 cm layer of thickness   This fact may be used to detect catalase in comparatively dilute solutions   A catalase preparation obtained from pumpkin seedlings by the method of Zeile (2) showed a low activity (k = 10)   Consequently the enzyme spectrum, which has been found in similar preparations of higher activity by Zeile, could not be seen directly with a pocket spectroscope in layers up to 50 cm   But after saturation with NO

the solution appeared red in 50 cm layers and showed the spectrum of the catalase NO complex given above   The extinction of the NO complex is also greater than that of the fluoride catalase com pound described in the preceding section   The combination of the enzyme with NO is reversible   By removing the NO from the solu tion by prolonged flushing with nitrogen, the spectrum of the free enzyme was completely restored   The regenerated enzyme was active   The linkage between the enzyme and nitric oxide may also be dissolved by the substrate   When hydrogen peroxide is added to the solution of NO catalase complex, the substrate is actively decomposed and the spectrum of the free enzyme is partially restored

The reaction between catalase and nitric oxide cannot be used to distinguish the enzyme spectrum from that of methemoglobin   The spectrum of NO methemoglobin is identical with the spectrum of NO-catalase, with the exception of an additional very faint band of the enzyme complex at 610 mμ Neither complex is appreciably dissociated by illumination with a 750 c p tungsten lamp

Effect of Acetylene

Acetylene does not combine with catalase   When a neutral enzyme solution of $k = 3410$ was saturated with pure acetylene, the spectrum appeared unchanged   Methemoglobin, at pH 6 9, was likewise found not to combine with acetylene   The same ap plies to hemoglobin prepared by reduction of methemoglobin with hydrosulfite at pH 6 9   A hemoglobin complex with acetylene has been described but its existence is doubtful (28)

Effect of Hydroxylamine

To 9 cc of a catalase solution of $k = 7125$ there was added 1 cc of 20 per cent hydroxylamine hydrochloride solution   When the spec trum of this solution in 2 cm layer was projected into the pocket spectroscope simultaneously with that of an untreated enzyme solu tion, no change in the spectrum could be detected within 20 hours A fine turbidity appeared and settled to form a colorless precipitate The stability of the enzyme spectrum toward hydroxylamine is of interest in view of the observation that this substance inhibits the activity of the enzyme (29)

Effect of Substrates on the Enzyme Spectrum

The formation of an unstable intermediate in the catalase-mono-ethyl hydrogen peroxide reaction and its absorption spectrum consisting of two bands in the green region have recently been described (3)

Attempts to observe a similar intermediate in the catalase-hydrogen peroxide reaction have so far been unsuccessful

There is no visible change in the spectrum of the enzyme when it is adsorbed on aluminum hydroxide gel or on silicic acid particles (30)   It has been shown that in these adsorbates the prosthetic group of the enzyme is free   It is available for the catalysis of hydrogen peroxide and of ethyl hydrogen peroxide   In the latter case the spectrum of the intermediate is observed   Furthermore, cyanide will produce the spectrum of the inactive catalase-HCN complex which has previously been observed in solution (1)

A few experiments were conducted in which the catalase-silicic acid adsorbates were suspended in non-aqueous solvents   It was hoped that the catalysis of hydrogen peroxide by this arrangement would be slowed down to such an extent as to render the spectrum of the hypothetic intermediate in this reaction visible   Acetone, ether, and glycerol were used for suspending the dried enzyme adsorbates   The spectrum of the enzyme appeared unchanged   On addition of ethyl hydrogen peroxide the spectral cycle already described could be observed   But with hydrogen peroxide as the substrate no change in the enzyme spectrum could be detected, although in some cases the rate of the catalysis seemed to be less than in the homogeneous system

Although these experiments did not have the result desired, they are of interest   They suggest that water as a solvent may not be indispensable for the enzymatic catalysis   In one experiment the dry adsorbate was suspended in anhydrous ether and the hydrogen peroxide-ether solution was likewise dried over sodium sulfate, but the possibility that a small amount of water was still present in the silicic acid gel cannot be excluded

On the Visibility of the Enzyme Spectrum in Liver Tissue

The absorption band of catalase in the red at 622 mμ (α-band) does not coincide with absorption bands of other normal constituents

of living mammalian tissues   The absorption bands of cytochrome a and the α band of Warburg's respiratory enzyme are situated near 600 mμ The catalase concentration in the liver of some animals, e g   horse and rat, is so high that an attempt was made to see the α band of catalase in these tissues   The main difficulty that pre sented itself is the high concentration of oxyhemoglobin in the liver Though the two main absorption bands of the blood pigment are centered at about 540 and 575 mμ, the latter band will stretch into the red region in high concentrations   Though it was possible to make slices of fresh horse liver of 1 cm sufficiently transparent for spectroscopic examination by the use of carbon arc lamps (25 am-peres), the absorption of the blood pigment made an observation of the enzyme band impracticable   The liver of a rat was therefore freed from blood as much as possible by perfusion with Ringer solu tion *in situ*[6]   The whole liver, which had assumed a yellow pink color, was placed in a 3 cm absorption cell and examined with a pocket spectroscope in the concentrated beam of a 500 watt projec-tion lantern   The bands of oxyhemoglobin were still visible, but no band of the enzyme in the red region could be seen   The liver was then ground up and placed in a 1 cm absorption cell   Under these conditions, there was a faint band visible near 630 mμ Upon add ing neutralized sodium cyanide solution, the band disappeared, and two bands at 600–590 and 560 mμ appeared instead   To another sample primary potassium phosphate and sodium fluoride were added   The band in the red was shifted to about 610 mμ As has been shown above, the catalase fluoride complex has two bands in the red, one at 620 and the other at 600 mμ But in crude liver extracts, where the enzyme spectrum may be observed (1), it was found that upon adding fluoride only the stronger short wave band of the two was visible   It is therefore not impossible that the band observed in liver tissue is the α band of catalase

DISCUSSION AND CONCLUSIONS

Catalase is resistant to oxidizing agents, e g , ferricyanide   It is also resistant to reducing agents, e g , catalytically activated hydro-

gen, hydrosulfite, ferrotartrate, cysteine   The hemin group of the enzyme will combine with cyanide, sulfides, nitric oxide, fluoride It will not combine with carbon monoxide   Catalase is therefore a *ferric* complex   The stability of the ferric iron in the enzyme toward reducing agents is not due to the structure of the porphyrin with which it is combined   This porphyrin is the protoporphyrin of the blood pigment   In combination with globin (methemoglobin) the ferric iron is readily reduced by the same reagents which have no effect on catalase   The stability of the ferric iron in the enzyme is

TABLE II

Absorption Maxima of Catalase and Derivatives

Compound	Position of absorption maxima	Reference
	$r\mu$	
Catalase	622, 540, 505, 409	
HF-catalase	620, 600, 540, 505	
NO-catalase	576, 541	
HCN-catalase	589, 557	(1)
H S-catalase	640, 580	(1)
C-H₅OOH-catalase	570, 534	(3)
Pyridine hemochromogen, derived from catalase	557, 527	(32)
CO pyridine hemochromogen	565, 530	
Hydrazine hemochromogen, derived from catalase	(622), 588, 545	
CO hydrazine hemochromogen	578, 542	

The spectra for which no reference is given have been determined in the present study
The Soret band in the far violet region has so far only been determined for the unmodified enzyme   It is very probable that the derivatives of the enzyme possess maxima in the same region

therefore due to the protein component   It may be that the type of hematin-protein linkage in catalase is the reason for this phenomenon The suggestion of Bersin (31), that sulfur may participate in this linkage is interesting but as yet, has no experimental basis

Hydrazine or pyridine and hydrosulfite convert catalase into hemochromogens containing ferrous iron   But in these hemochromogens the hematin is no longer attached to the protein   This has been replaced by the nitrogenous bases hydrazine and pyridine   Both hemochromogens combine reversibly with carbon monoxide   Photo-

dissociation has only been demonstrated in the case of the pyridine hemochromogen

The positions of the absorption bands of catalase and its derivatives are listed in Table II

The main absorption band (Soret's band) of hemin complexes with nitrogenous substances (nitrogen bases, proteins) is situated at the border between the visible and the ultraviolet region of the spectrum It has now been found that the spectrum of purified liver catalase has a well defined maximum of high extinction in this range, at 409 mμ This is further evidence for the hemin nature of the enzyme

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SUBSTANCES AFFECTING ADULT TISSUE IN VITRO

III A STIMULANT (THE "A FACTOR") IN SERUM ULTRAFILTRATE INVOLVED IN OVERCOMING ADULT TISSUE DORMANCY

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INTRODUCTION

Pieces of adult thoracic chicken aorta planted in a medium of diluted plasma will, after a lag period of 3 to 5 days, begin to grow (1)   It is not known why there is a lag period, nor is it known why this tissue should grow *in vitro* when imbedded in the same plasma to which it had been exposed, without growing, in the animal   No embryo extract or other foreign material need be present (dilution of the plasma is also unnecessary)   The stimulus to growth might result from one or more of the following injury to the tissue, or the presence of clotted fibrin, or the presence of some stimulant contained in the serum portion of the plasma clot

This paper will show that the plasma contains an agent (the "A factor") which plays a rôle in overcoming the dormancy of fresh adult tissue

EXPERIMENTAL

Details of our methods for testing the effects of substances on the initial growth of adult tissue are discussed elsewhere[1] (1–2)   The experiments were controlled with tissue incubated in Tyrode solution, and usually also with fresh untreated tissue

[1] During the 3 day incubation of tissues in various fluids (previous to planting in a plasma medium) it was found necessary to keep the pH at about 7 4 by use of CO_2 air mixtures (in a stoppered tube)   Adjustment of the pH with HCl resulted in less than one-quarter the stimulation obtained in the presence of CO_2

is placed over the bag holder A and onto the No 2 rubber stopper   Three No 8 rubber bands are used to hold the bag in place   These are first placed on a piece of glass tubing which can be slid over the bag, and are then slipped into place. Bag holder A (with the bag) is then placed on tube B   50 to 75 ml of dog serum is poured into cylinder C, then permitted to run into B by opening stop-cock D The rubber tubing from mercury trap F is connected to E   Stop-cock D is closed, G and H are opened and the pressure is raised, with leveling bulb I, to about 50 cm of Hg   (If the air capacity of the apparatus is too great for the volume of mercury, G should be closed while I is first lowered to let air enter through manometer J, then raised until the mercury rises in the manometer) The motor[2] is then started and stop-cock H is closed

The motor serves to rock the mercury trap F ten times a minute   This causes the serum to surge violently along the surface of the membrane, thus preventing accumulation of proteins on the membrane   As the ultrafiltrate is collected through tube L escape of CO_2 is impeded by the aluminum cover M   Since the serum is under positive pressure it does not lose enough CO_2 to cause precipitation of the A factor

More serum can be added after closing stop-cock G, opening H, opening D carefully, then disconnecting rubber tubing at E   One bag can be used for 150 ml of serum

The ultrafiltrate is placed in a stoppered flask.   1/100 volume of 0 5 per cent phenol red is added   The pH is made slightly acid with CO_2 For sterilization a cotton plug is substituted for the stopper and the flask is placed in boiling water 10 minutes   It is then cooled, the neck flamed, a sterile rubber stopper is introduced, and more CO_2 is added, aseptically, to bring the pH to 7 0 or 7 2   The ultrafiltrate is then kept in the refrigerator

Electrodialysis —The electrodialysis of the serum ultrafiltrate was performed in the apparatus shown in Fig 2   10 ml of dog serum ultrafiltrate was placed in the neutral compartment   The anode and cathode compartments each contained 2 5 ml of Tyrode solution in a collodion bag   These two compartments were each connected by an agar (2 per cent agar in Tyrode) bridge to an electrode cup Tyrode solution containing phenol red was placed in the electrode cups   The anode was a platinum wire and the cathode a nichrome wire   Current from a 110 volt D C circuit was passed for 1 5 hours through a 10 watt lamp in series with the outfit   During the electrolysis 1 N NaOH was added to the anode cup and 1 N HCl was added to the cathode cup, a little at a time to keep the cup solutions neutral

After electrodialysis for 1 5 hours samples were drawn from each compartment, sterilized at 100 C for 5 minutes, and tested

Preparation of Copper Precipitates of the A Factor —1 2 ml of a 7 5 per cent

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[2] The same motor is used simultaneously on this machine and on the membrane machine and dialyzer   It is a 1/500 H.P reduction gear motor with reduced speed of 10 R.P M., sold by the Bodine Electric Co , Chicago

precipitations was treated with copper acetate giving 1 6 gm of precipitate This, when freed from the copper, was inactive

The A Factor from Urine —20 ml of human urine was treated with 1 6 ml of 5 per cent CaCl₂ solution, plus 0 25 ml of 0 5 per cent phenol red, plus 6 0 ml of 0 1 м NaOH The precipitate was separated by centrifuging, and dissolved in 10 ml H₂O plus 1 ml 0 1 м HCl (The solution was cloudy and was still cloudy after adding 0 5 ml more HCl)

TABLE I

Stimulating Activity of Dog Lymph (Diluted with Two Parts and with Eight Parts of Tyrode Solution Respectively) (Exp 61D)

Chicken aorta incubated 4 days before planting in	Growth values after planting			Relative growth on 3rd day
	1 day	2 days	3 days	
Tyrode (control)	0	0	6	1
Dog lymph plus 2 parts Tyrode	1 0	13	32	5
Dog lymph plus 8 parts Tyrode	0 1	4	18	3

TABLE II

Stimulating Action of Dog Serum Euglobulin (Separated by Dialysis and Concentrated Ten Times) As Compared with Chicken Serum (Diluted to One Third) (Exp 35C)

Tissue incubated 3 days before planting in	Growth values after planting				Relative growth on 4th day	Growth divided by con ce tra tion
	1 day	2 days	3 days	4 days		
Chicken serum (×⅓)	0	0	5	13	1	28
Dog serum euglobulin (×10)	0	0 1	5	14	1 1	1

The A factor was re precipitated with 1 6 ml CaCl₂ solution plus 1 5 ml NaOH, and centrifuged This precipitate was suspended in 4 ml Tyrode solution and adjusted to pH 7 0 with 10 per cent CO₂ A precipitate which formed was centrifuged off, suspended in Tyrode solution, and tested It was found to be stimulating, but the fluid from this last precipitation was found to be even more stimulating Neither appeared to contain toxic substances

Since the A factor is heat resistant the various fractions were sterilized by heating at 100 C for 5 minutes after making the pH acid with CO₂

DISCUSSION OF RESULTS

Serum Stimulation —The action of serum in overcoming adult tissue dormancy was tested by incubating pieces of adult chicken aorta in

chicken serum for 3 days previous to planting the tissue in a dilute plasma medium   Fig 3 shows that the lag period after this serum treatment was much less than that of the control tissue, which was incubated in Tyrode solution   The reduction in lag period is accompanied by an increased initial growth rate   This has been repeated many times with the same result   Apparently part of the change in the tissue taking place during the lag period is brought about in serum, but not in Tyrode solution, at 37°C   This suggested that serum contains a substance involved in overcoming adult tissue dormancy [3]

It should be emphasized that this paper is concerned with the process of *overcoming the dormancy* of fresh adult tissue—not with the growth of active cells, as in embryo cultures, or in cultures of adult cells after growth is under way

Serum diluted with two parts of Tyrode solution often stimulated better than undiluted serum   Daily renewal of the serum in which the tissue was incubated gave no better stimulation than the use of the same serum continuously for 3 days There would appear to be an adequate supply of the supposed stimulant in serum The volume of fluid was about 100 times that of the tissue   Addition of heparin to serum decreased its activity

The stimulating action of serum is *not species specific*   Dog serum stimulated chicken tissue as well as chicken serum did, in spite of the foreign proteins (Fig 3)

Lymph and Ventricular Fluid —Dog lymph was found to be highly stimulating to the growth of adult chicken aorta, even when diluted with eight parts of Tyrode solution (Table I)   Human ventricular fluid showed some stimulative activity when undiluted   The activities of these fluids withstood sterilization at 100°C   for 5 minutes

Serum Proteins —The stimulating effect of serum on dormant adult tissue cannot be due to the serum albumin since this protein is inhibitory to growth, as is shown in Fig 4   It is also seen that concentrated whole globulin, precipitated with ammonium sulfate, then dialyzed, is somewhat stimulating   The same is true of euglobulin (separated by dialysis and concentrated ten times (Table II))   However, in normal concentration the globulin can account for only a fraction of the stimulating action of whole serum on dormant tissue   See last column of Table II

[3] *No embryo extract was present in these cultures*   It had little effect on dormant adult tissue (6), in fact it was sometimes inhibitory
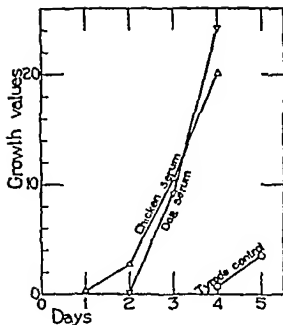
FIG 3 Stimulation produced by incubating chicken aorta tissue in serum (from dog or chicken) for 3 days previous to planting in chicken plasma The control tissue was incubated in Tyrode solution (Exp 34A)
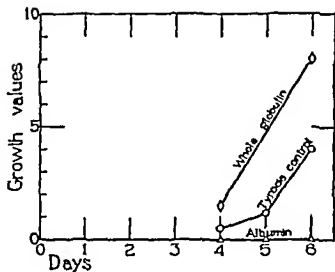
FIG 4 Slight stimulating action of concentrated serum globulin, and inhibitory action of serum albumin (Exp 36A)
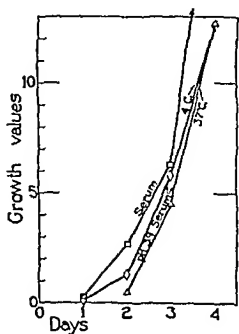
FIG 5 The stimulating activity of serum after keeping it at pH 3 9 for 24 hours at either 4 C or 37°C (Exp 33B)
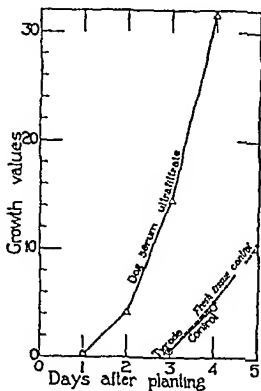
FIG 6 Stimulation of chicken tissue incubated 3 days in dog serum ultra filtrate (Exp 39B) Note that ultrafiltrate reduced the lag period to 1 day while the Tyrode treated tissue (solid line) was almost identical with the untreated fresh tissue (dotted line)

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Serum Lipase —The stimulating action of trypsin on fresh adult tissue (1) suggested that some enzyme in serum might act on dormant tissue   The anti-tryptic action of serum would preclude trypsin, but serum lipase remained as a possibility

The lipase in serum is largely destroyed (4) at pH 3 9 at 37°C for 1 day   We treated serum in this manner, readjusted it to pH 7 5, and diluted it with Tyrode solution to three times its original volume   Fig 5 shows but slight difference between this and a control serum kept at 4°C   This slight decrease of activity can be accounted for by loss of the A factor (see below)   If serum lipase does exert a stimulating effect on dormant tissue it is very slight

This conclusion is supported by experiments with lipase purified from raw pancreas   Tissue treated with this lipase failed to grow   It was prepared and tested by the methods of Willstatter and Leitz (5)

Further evidence against the involvement of serum enzymes is the resistance of the stimulating activity to heat   Serum heated at 60°C for an hour lost none of its activity   At 100°C for 30 minutes there was only a slight loss in activity providing the pH was kept below 7 8 by use of CO_2-air mixtures

Serum does not act at ice box temperature as does trypsin (1)

Ultrafiltrate from Serum —Since the action of serum in overcoming adult tissue dormancy appeared not to be associated with the proteins or enzymes of serum it remained to test the ultrafiltrate   The ultra-filtrate from serum (obtained by forcing serum under positive pressure through a collodion bag) was found to be highly stimulating to the growth of fresh adult chicken aorta tissue[4] (Fig 6)   Its activity seemed to be sufficient to account for all the stimulation by the serum Furthermore the residue, largely serum proteins, which does not pass through the collodion membrane was not stimulating (after thorough dialysis followed by equilibration against Tyrode solution)

Electrodialysis —Serum ultrafiltrate was subjected to electrodialysis using collodion bags to separate the anode and cathode fluids from

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[4] In Fig 6 it will be noted that there are two control tissues, one incubated in Tyrode solution, the other was fresh untreated tissue   The growth was almost identical in both cases, showing that incubation in Tyrode solution preserved the dormancy of the tissue with little damage   That this tissue was well preserved in Tyrode solution was also shown by histological sections   Hence the stimulation produced by the ultrafiltrate as in Fig 6 certainly cannot be ascribed to mere preservation of the tissue

To avoid confusion, the fresh tissue control was not plotted in every case   It is inserted in Fig 10, where there was no Tyrode control   In general the two controls were nearly alike (Fig 6)
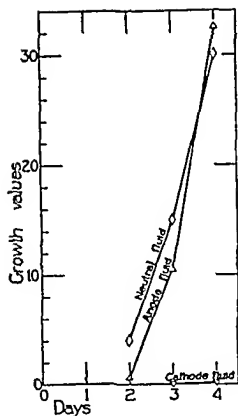
FIG 7

FIG 8

FIG 7 Stimulating activity of the anode fluid, as contrasted with the cathode fluid (Exp 37B)
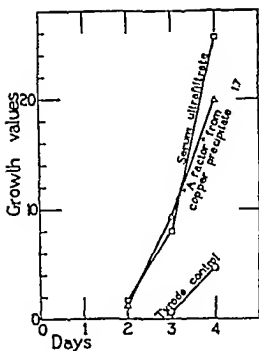
FIG 8 Stimulating activity of material recovered from a copper precipitate (Exp 39B)
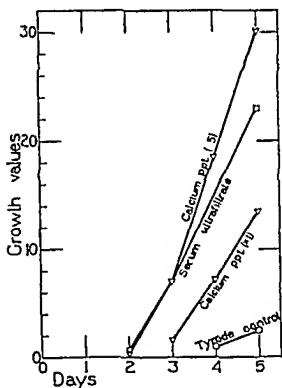
FIG 9

FIG 10

FIG 9 Stimulating activity of a calcium precipitate from serum ultrafiltrate (Exp 42A)
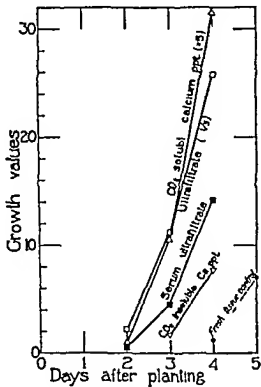
FIG 10 Stimulating activity of the CO_2 soluble fraction of a calcium precipitate (Note that the diluted ultrafiltrate was more stimulating than the undiluted (Exp 46D))

the ultrafiltrate in the central compartment, as shown in Fig 2   The anode fluid was shown to be quite stimulating to dormant tissue while the cathode fluid gave a negligible stimulation (Fig 7)   This was repeated with similar results   It was therefore concluded that the substance is an acid

Copper Precipitate of the A Factor —A copper precipitate from serum ultrafiltrate was prepared as described above   It was freed from the copper, giving a water-soluble material which was quite stimulating to the initial growth of adult tissue (Fig 8)   Accurate estimation of the activity is difficult but that recovered from the precipitate appeared to be about 50 per cent of the original total activity   However, the possible toxicity of remaining traces of H_2S made it advisable to try other methods of precipitation

Calcium Precipitation —A precipitate was obtained from serum ultrafiltrate by adding calcium chloride in slightly alkaline solution This precipitate when suspended in Tyrode solution was definitely stimulating to dormant aorta tissue, as shown in Fig 9   Here too the yield is hard to estimate   We can merely say that "20 per cent or more" of the original total activity was recovered (although contained in a smaller volume)   The presence of calcium, unlike the copper, did not appear to interfere with the testing

Attempted Purification —Attempts to obtain more active fractions were not satisfactory   When the ultrafiltrate was treated with calcium chloride, 85 per cent of the precipitate formed at pH 7 3 and the remaining 15 per cent precipitated at pH 8   The first precipitate seems to contain more activity per gram than the second   This warranted discarding the latter   When the first precipitate was suspended in Tyrode solution and this was made acid with pure CO_2, most of the precipitate went into solution   The "CO_2 soluble" fraction contained most of the activity (Fig 10) but we are not certain that its activity on a dry weight basis is greater than that of the insoluble portion

Distillation of Ultrafiltrate —Several attempts were made to concentrate the A factor by vacuum distillation of the ultrafiltrate   The distillate contained no activity   Various fractions obtained from the residue were mostly inactive   No activity was obtained from alcohol-soluble or from acetone-soluble fractions   The water-soluble fractions

TABLE III

Stimulating Activity of Material from Urine Twice Precipitated as Described in Text (Exp 42B)

Tissue incubated 3 days before planting in	Growth values after planting					Relative growth on 4th day
	1 day	2 days	3 days	4 days	5 days	
Tyrode (control)	0	0	0 1	0 5	2 5	1
Urine fraction (×5)	0	0	3	9	17	7

TABLE IV

Effect of Heating Dog Serum Ultrafiltrate at 100°C for Different Intervals, with and without the Presence of Air (Exp 36B)

Tissue incubated 3 days in ultrafiltrate previously heated as indicated	Growth values after planting			
	1 day	2 days	3 days	4 days
Ultrafiltrate 100 C 10 min	0	1	9	22
Ultrafiltrate 100 C 3 hrs in CO_2-air	0	0	0 8	3
Ultrafiltrate 100 C 3 hrs in CO_2-N_2	0	0	0 3	4

TABLE V

Destruction of Serum Ultrafiltrate Activity by Heating at 100°C 5 Minutes with 1/30th Volume 1 M HCl (Afterwards Neutralized) (Exp 38B)

Tissue incubated 3 days, before planting in	Growth values after planting				Relative growth on 4th day
	1 day	2 days	3 days	4 days	
Serum (×⅓)	0	1	4	13	9
Ultrafiltrate heated with HCl	0	0	0	1 5	1

TABLE VI

Destruction of Serum Ultrafiltrate Activity by Heating at 100°C 5 Minutes with 1/30th Volume 1 M NaOH (Afterwards Neutrali ed) (Exp 39C)

Tissue incubated 3 days before planting in	Growth values after planting				Relative growth on 4th day
	1 day	2 days	3 days	4 days	
Tyrode solution	0	0	0 2	2	1
Serum (×⅓)	0	1	7	15	10
Ultrafiltrate heated with NaOH	0	0	0 6	1 5	1

were also inactive   In one experiment, however, a suspension of an insoluble residue was slightly stimulating

In view of the stability of the A factor this apparent loss of activity may seem surprising   However, a sample of ultrafiltrate which had been concentrated to dryness by vacuum distillation was dissolved in its original volume of water and found to have nearly its original activity   The apparent inactivity of the various fractions mentioned above may have been due to our failure to test these fractions in solutions having the correct concentrations of electrolytes and glucose as well as suitable pH, osmotic pressure, and CO_2 tension

The A Factor from Urine —Because the A factor passes so readily through a collodion bag, it should be expected to pass through the kidneys into the urine   Urine was not tested directly but was partially purified as described above, by precipitating twice as the calcium salt The portion of this product which was soluble at pH 7 0 (in the presence of bicarbonate) was found to be fairly stimulating in a volume one-fifth that of the original urine sample (Table III)

Although urine may later prove to be the best source of the A factor, we find it more convenient at present to use serum ultrafiltrate, without purification, as a stimulating fluid with which to treat our tissues, or to wash our cultures   It also serves as a basic medium in our sterile perfusion pump

Properties of the A Factor —From the above data it may be seen that the A factor has a small molecular size (since it passes through a collodion membrane) and furthermore the first and last portions of ultrafiltrate seemed to have about equal activities   It is an acid   It can be precipitated as a copper or calcium salt

The A factor is not appreciably inactivated at 100°C for 10 minutes (providing it is kept neutral), but 100°C for 3 hours caused loss of activity even in the absence of O_2 (Table IV)   Heating 5 minutes at 100°C at about pH 2 or pH 12 destroyed all the activity (Tables V and VI)

It is not species specific, chicken tissue being stimulated by serum ultrafiltrate from chickens, dogs, and rabbits

If kept neutral in the ice box, ultrafiltrate remains active at least 10 months   Chicken plasma has induced growth of fresh tissue after 11 months storage

COMMENTS

The A factor seems to be the principal one of the stimulating agents in adult blood plasma by virtue of which plasma serves to overcome the dormancy of adult tissue *in vitro*   It is not claimed that the A factor is the only agent in serum which is involved in this process, nor that it is a single substance   This action of the A factor on dormant tissue must not be confused with the stimulation of active cells (although the A factor seems to aid them also)   It will be brought out in a later paper (6) that substances which stimulate embryo cell cultures do not necessarily affect the dormant state of adult tissue (but may stimulate the adult cells once growth is under way)

Tests have been made on numerous known substances (6), including hormones, vitamins, amino acids, proteins, protein digestion products, urea, uric acid, glutathione, allantoin, etc   Not any of the substances so far tested have the properties of the A factor, or can take its place Some of them (such as amino acids and thyroglobulin, but not thyroxin) augment the action of the A factor

We do not know whether the A factor acts directly upon the tissue inhibitor (2)   There is evidence both for and against this explanation We have been able to induce growth in adult tissue only in the presence of the A factor, and therefore consider it essential for growth   But we do not feel that it alone is responsible for overcoming dormancy Some other change in the tissue must be involved—perhaps as a result of mechanical injury or necrosis

SUMMARY

1   The growth of fresh adult tissue in plasma medium suggested that plasma contained a substance active in overcoming the dormancy of adult tissues

2   Incubation of adult chicken aorta in serum (from chickens or other species) before it was planted in a plasma medium, resulted in a much shorter lag period and a faster initial growth than that of control tissue incubated in Tyrode solution (or of fresh untreated tissue)   In other words, serum helped overcome the dormancy of adult tissue while Tyrode solution preserved the tissue in a dormant state   This activity of serum was shown to be due neither to its albumin or globulin nor to its lipase or other enzymes

3 The ultrafiltrate from serum was highly active in overcoming adult tissue dormancy, while the dialyzed residue was inactive The ultrafiltrate was not species specific It withstood 100°C in neutral solution for 10 minutes, but not for 3 hours, and was destroyed at pH 2 and pH 12 It was active after 10 months in the ice box It does not affect tissues in the cold

4 Copper acetate precipitated a fraction from serum ultrafiltrate which, when freed from copper, was stimulating to the initial growth of adult aorta tissue

5 Calcium chloride precipitated fractions from serum ultrafiltrate (and also from urine) which were stimulating

6 Attempts to concentrate the A factor, the active agent, by vacuum evaporation and subsequent fractionation with different solvents were not satisfactory

7 Lymph was highly stimulating and ventricular fluid slightly stimulating

8 No growth was obtained in the absence of the A factor, but we doubt that it is alone responsible for overcoming the dormancy of adult tissue

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CARBOXYPEPTIDASE

I The Preparation of Crystalline Carboxypeptidase[*]

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Carboxypeptidase (CP) splits the amide linkages of certain amino
acid compounds such as chloracetyl tyrosine, tyrosyl tyrosine, and
leucyl glycyl tyrosine   In each case an amino acid is liberated which
in the intact compound has a free carboxyl group   Since chloracetyl
tyrosine,

$$Cl\ CH_2CO-NH$$
$$|$$
$$HOC_6H_4H_2C\ CHCOOH$$
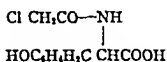
is attacked, it is clear that the substrate of carboxypeptidase need not
have a free amino group nor need it, despite the name of the enzyme,
be a peptide   It has been assumed, but not proven experimentally,
that chloracetyl tyrosine is attacked by only a single enzyme and
that the same enzyme which attacks chloracetyl tyrosine also attacks
the other supposed substrates of carboxypeptidase   It may be,
however, that what has hitherto been called carboxypeptidase is in
reality a mixture of enzymes   The previous work on carboxypeptidase
has been reviewed by Waldschmidt-Leitz (1931) and Bergmann
(1934)

The present paper (Part I) describes the preparation from autolyzed
beef pancreas of a crystalline, water insoluble protein which attacks
chloracetyl tyrosine and peptic digests of proteins   This crystalline
carboxypeptidase is active even in the presence of formaldehyde which
abolishes the free amino groups of both enzyme and substrate   Car-

boxypeptidase is the only proteolytic enzyme of those which have been crystallized which attacks simple substrates of known structure The two following papers describe (Part II) the extraction from fresh pancreas of the inactive precursor of carboxypeptidase, pro-carboxypeptidase, its partial purification, and its activation by trypsin, and (Part III) the estimation of carboxypeptidase and of its inactive precursor

Two sources of material have been used for the preparation of crystalline carboxypeptidase, the turbid fluid which exudes when sliced frozen bovine pancreas is thawed overnight at 5°C,[1] which is not always available in large quantities from commercial sources, and ordinary commercial frozen pancreas, which can be obtained from any of the large meat packers The turbid fluid is by far the more convenient starting material 1 liter of this fluid yields roughly a gram of crystals

The turbidity of the fluid which exudes when frozen pancreas is thawed is due to a small amount of dark, slimy material which at the pH of the fluid cannot readily be removed by filtration If the fluid is acidified (green to brom cresol green) and warmed, the dark, slimy material clots and can then readily be filtered off The filtrate on dilution with water yields a precipitate which contains most of the carboxypeptidase and most of the proteinase of the original fluid This precipitate is only partially soluble in barium hydroxide in slightly alkaline solution (pink to phenolphthalein) When the soluble part, which contains all the carboxypeptidase, is neutralized (orange to phenol red) a protein crystallizes out which attacks chloracetyl-tyrosine (Fig 1) On repeated recrystallization this protein becomes free of proteinase but retains all its original carboxypeptidase activity

When crystalline carboxypeptidase is prepared directly from commercial frozen pancreas the early stages of the preparation are modified An acidified sodium chloride extract is filtered, the filtrate is 0 6 saturated with ammonium sulfate, and the precipitate formed is dialyzed against water The fraction which remains precipitated after dialysis is treated with barium hydroxide

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[1] This same material was originally used for the isolation of trypsin (Northrop and Kunitz (1932–33))

The specific activity of crystalline carboxypeptidase is

$$0\,081\,[CP\ u\,]_m^{CT}{}_N\quad\text{and}\quad 0\,103[CP\ u\,]_{mg\ N}^{PDE}$$

The specific activity of twice crystallized carboxypeptidase is not

FIG 1 Carboxypeptidase crystals × 85

changed by repeated or fractional recrystallization If crystalline carboxypeptidase is partially denatured and precipitated, the surviving

[2] Part III (*J Gen Physiol* in press) will describe the estimation of carboxy peptidase and define the carboxypeptidase unit (CP u) Two substrates are used for the estimation of carboxypeptidase chloracetyl tyrosine (CT) and a for molized peptic digest of edestin (PDE)

soluble protein has the same specific activity as the original crystalline material

Recrystallized carboxypeptidase gives no test for proteinase, dipeptidase, amino polypeptidase, and amylase

The elementary analysis of carboxypeptidase is C, 52 6 per cent, N, 14 4 per cent, H, 7 2 per cent, S, 0 47 per cent, P, 0 00 per cent, ash, 0 68 per cent

An amount of carboxypeptidase containing 0 20 mg N gives the same color with the phenol reagent as 0 15 mg tyrosine

Preparation from Pancreatic Fluid

The Starting Material.—The pancreatic fluid which exudes when frozen pancreas is thawed consists mainly of proteins and protein split products and is highly variable in composition   As a result the absolute amount of crystalline carboxypeptidase that is obtained finally, and the yields of carboxypeptidase at each step, are also highly variable   If the directions are followed, however, a large crop of crystals is obtained without fail   It is not necessary to measure the carboxypeptidase content of the original fluid which is usually around 0 07 $[CP u]_{ml}^{PDL}$

The First Globulin Precipitate.—5 N acetic acid is added with stirring to the crude fluid until the solution is definitely green to brom cresol green   The acidified solution in 4 liter Erlenmeyer flasks is left in a 37°C bath for 2 hours and then filtered   The filtrate contains all the carboxypeptidase   Ten volumes of tap water are added to each volume of filtrate in large glass vessels   A precipitate forms which settles   At the end of the day the supernatant fluids are siphoned off and rejected and the precipitates are collected in a single vessel   The next morning the supernatant is again siphoned off and rejected and the precipitate is filtered on Schleicher and Schull folded filter paper No 588, 50 cm diameter   The smaller and also thinner paper of the same number is less satisfactory   Finally the precipitate is evenly suspended in enough water to give a volume roughly one-fifth of the original volume of pancreatic fluid   A sample is dissolved with a little di-potassium phosphate and sodium chloride and its activity is measured   From 35 to 90 per cent of the original activity is recovered in the first globulin precipitate

Barium Hydroxide Extraction.—The suspension of the first globulin precipitate is diluted to have roughly 0 25 $[CP u]_{ml}^{PDL}$ 0 2 M barium hydroxide is added with vigorous stirring until the solution is definitely pink to phenolphthalein and the suspension is immediately filtered with Standard Super-Cel[3] on a Buchner

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[3] Standard Super-Cel is the trade name of a particular Celite filter aid of medium porosity   Hyflo Super-Cel is a coarser grade, Filter Cel a finer   Celite filter aids are made from diatomaceous earth (kieselguhr) by Johns Manville, 22 East 40th Street, New York City   The names of the European sales agents and booklets describing the Celite filter aids may be obtained from the manufacturer

funnel or centrifuged Barium hydroxide dissolves only a part of the globulin precipitate whereas sodium hydroxide at the same pH dissolves it completely If not enough barium hydroxide has been added the supernatant solution obtained by centrifugation is turbid and does not contain all the carboxypeptidase If too much barium hydroxide is added the carboxypeptidase activity is slowly de stroyed As a precaution, when a large batch of material is being worked up, the barium hydroxide extraction is first carried out on a small scale, the amount of barium hydroxide added is recorded, and the activity of the solution is meas ured after removal of the undissolved protein The solution should be clear and its activity should be the same as that of a complete solution of the globulin precipitate obtained by adding di potassium phosphate and sodium chloride instead of barium hydroxide

Crystallization —Immediately after centrifugation, 1 N acetic acid is added to the barium hydroxide solution with vigorous stirring until the solution becomes just turbid (orange to phenol red) Crystals appear slowly if the solution is simply allowed to stand In practice the solution is heavily seeded, allowed to stand the rest of the day at room temperature with occasional stirring and finally left in the refrigerator overnight The next morning the supernatant solution is siphoned off and rejected and the crystals which have settled to the bottom of the vessel are centrifuged The supernatant solution is rejected, the crystals are again suspended in water and again centrifuged 40 to 80 per cent of the car boxypeptidase of the barium hydroxide extract is obtained in crystalline form

At the pH used for crystallization carboxypeptidase is stable, its crystalline form is insoluble and its amorphous form is soluble At a more acid pH (green to brom cresol green) both the crystalline and amorphous forms are insoluble and so since precipitation of the amorphous form is rapid compared with crystalliza tion, carboxypeptidase is immediately and completely precipitated in the amor phous form If the amorphous precipitate is dissolved at the acid pH with a solution of sodium chloride, the carboxypeptidase slowly coagulates and is inacti vated It is clear that carboxypeptidase is insoluble over a wide range of pH and that the proper pH for crystallization is far on the alkaline side of the pH of mini mum solubility

Recrystallization —To a suspension of crystals having about 1 0 [CP u]$_{\text{ml.}}^{\text{PDE}}$ (the exact concentration is not important) 0 1 N sodium hydroxide is added slowly with vigorous stirring until almost all the material is dissolved The undissolved material is removed immediately by centrifugation and 1 N acetic acid is immedi ately added to the supernatant solution until the first turbidity appears The solution is then seeded and allowed to stand several hours and the crystals centri fuged The total activity of the recrystallized protein is 80 to 100 per cent of the activity of the protein used for recrystallization The crystals are stored in the cold with toluol as a preservative and so far as I know are stable indefinitely in aqueous suspension If pure carboxypeptidase is dried more or less of the protein is converted into an insoluble, inactive form, even if the protein is dried while frozen or if the water is removed with sodium sulfate Impure carboxypeptidase, however can be dried with sodium sulfate without any loss of solubility or activity

This protective action of impurities has been noted with pepsin and many other proteins

It is important when adding sodium hydroxide not to make the solution too alkaline   The crystals sometimes dissolve very slowly and the process of solution should not be hurried by adding sodium hydroxide in excess   After the carboxypeptidase has been once recrystallized it usually dissolves completely when the solution is definitely alkaline to phenolphthalein   This pH is entirely safe   It is not important that the crystals be completely dissolved   Undissolved crystals can be saved and added to the supply of once crystallized material

Changes in Specific Activity —In general the variable specific activities ($[CP u]_{mg\ N}$) of the various fractions were not measured since they need not be known for preparative purposes   Typical values are given in Table I

Alternative Method of Preparation —If the first globulin precipitate is dissolved with basic potassium phosphate and heated to 60°C   under

TABLE I

	Pancreatic fluid	Acetic acid filtrate	Dilution precipitate	Barium hydroxide extract	Once crystallized CP	Twice crystallized CP
$[CP u]_{mg\ N}^{PDE}$	0 00277	0 00265	0 0128	0 0222	0 0814	0 103

suitable conditions much of the protein is digested and the remaining protein has a very high carboxypeptidase and a very low proteinase activity   But I have never succeeded in obtaining crystals or in obtaining a preparation of carboxypeptidase completely free of proteinase without a fractionation with barium hydroxide

Preparation from Frozen Pancreas

Frozen beef pancreas is ground and stirred up with 3 times its weight of 2 per cent sodium chloride and 20 per cent of its weight of toluol (Eastman practical), and allowed to stand at room temperature   The next morning the fat and toluol are skimmed off and the suspension is filtered through gauze, 5 N acetic acid is added to the filtrate until it is green to brom cresol green   The acid solution is filtered on a Buchner funnel with the aid of Standard Super-Cel (Johns-Manville) and 390 gm ammonium sulfate are added to each liter of filtrate   The precipitate formed is filtered off and dialyzed overnight against cold water in a shaking dialyzer (Kunitz and Simms (1927–28))   The dialyzed solution is centrifuged,

the supernatant is discarded, and the precipitate is then treated like the precipitate obtained from the fluid which exudes when frozen pancreas is thawed

Recrystallization and Fractional Crystallization —A sample of twice crystallized carboxypeptidase had a specific activity of 0 082[CP u]$_{mg N}^{CT}$, of six times crystallized, 0 083[CP u]$_{mg N}^{CT}$ A sample of twice crystallized carboxypeptidase had a specific activity of 0 095[CP u]$_{mg N}^{PDE}$ It was recrystallized The first 25 per cent which crystallized out had a specific activity of 0 104[CP u]$_{mg N}^{PDE}$

Fractional Heat Coagulation —A test tube containing a solution of twice crystallized carboxypeptidase in half saturated sodium chloride solution containing 1 84 mg nitrogen per ml was placed in 52°C water for 3 minutes and the suspension was then cooled and filtered The filtrate contained 1 26 mg nitrogen per ml The specific activity of the carboxypeptidase in the filtrate was 0 104[CP u]$_{mg N}^{PDE}$

Fractional Denaturation by Acid —To a solution of twice crystallized carboxypeptidase in 5 per cent sodium chloride containing 1 06 mg nitrogen per ml was added an equal volume of a solution containing 3 parts 0 2 M sodium acetate to 1 part 0 2 M acetic acid The solution was kept 90 minutes at 37°C , cooled, and the denatured and precipitated protein filtered off The filtrate contained 0 26 mg nitrogen per ml The specific activity of the carboxypeptidase in the filtrate was 0 092[CP u]$_{mg N}^{PDE}$

Other Enzymes —Six times crystallized carboxypeptidase contains a trace of proteinase which can be detected by long digestion of hemoglobin (Anson and Mirsky (1933–34)) with a large amount of carboxypeptidase This slight activity could be accounted for by an impurity of 1 part trypsin to 30,000 parts carboxypeptidase

Thrice crystallized carboxypeptidase is free of dipeptidase and amino polypeptidase Fruton[4] found that 1 mg carboxypeptidase per ml produced no detectable digestion of 0 05 M dl leucyl glycine or dl leucyl glycyl glycine at pH 7 8 in 23 hours at 40 C

Six times crystallized carboxypeptidase is free of amylase A 1 per cent neutral solution of starch gave almost the original color with iodine after 24 hours at 37°C despite the presence of 0 7 mg per ml of carboxypeptidase

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[4] Dr Joseph Fruton, private communication

A METHOD FOR THE INVESTIGATION OF ELECTROSTENOLYSIS*

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INTRODUCTION

The essential structural and physicochemical conditions underlying the electrical sensitivity of living protoplasm are still imperfectly understood. It is known that when an irritable tissue is traversed by an electric current, chemical reactions are set up in the tissue, these reactions furnish the free energy for the response. The general physicochemical problem has reference to the conditions under which the passage of current through a polyphasic system, consisting of a combination of aqueous solutions and non aqueous phases in certain structural relationships, gives rise to chemical change. There are two conspicuous classes of such systems. (A) batteries or electrolytic cells, and (B) irritable living systems. Certain artificial systems of the type considered below form a third class (C). In general we find that no reaction occurs (apart from possible effects dependent on temperature) during the passage of a current through a homogeneous conductor, metallic or electrolytic. In the systems of class A, consisting in a combination of the two types of conductor, reaction does occur, but only when electric current passes between the two phases. The reaction is a boundary phenomenon, oxidation occurs where the current (positive stream) passes from the metallic phase (electrode) to the solution, and reduction where it passes in the reverse direction. For continuous electrolysis a certain critical potential (decomposition potential) is required, and a definite quantity of chemical change is associated with a definite quantity of current (Faraday's law). In living systems (class B) electrical sensitivity is bound up with a special type of structure in which electrolyte solutions containing oxidizable mate

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nals are separated from one another by thin partitions or membranes, apparently mainly non-aqueous in composition, having semi-permeability and high electrical resistance [1] These physical properties imply high electrical polarizability, and the connection between polarization and electrical stimulation has long been recognized   A similar connection between polarization and activation exists in the system, passive iron in nitric acid (belonging in class A), here activation is a simple case of electrolysis [2] In the living system also under certain conditions (brief duration of stimulating current) there is a connection between activation and the passage of a definite quantity of electricity through the irritable element [3] Living systems, however, have no metallic conductors, unless we admit the speculation that carbon chains set side by side may act as such conductors and so build up structures having the properties of electrodes   It seems more likely that the living system responds to electrical stimulation essentially because its type of structure furnishes conditions favorable to electrostenolysis or processes of a related kind   In stimulation a change in the electrical polarization of membranes is the critical event, in some way this initiates chemical reactions   The rules of polarity, of critical or threshold potential, of critical duration of flow, apply to both the living and the non-living types of electrically responsive system [2] Because of the possible relation of electrostenolysis to the general problem of stimulation it has seemed desirable to make a further and as far as possible quantitative study of this phenomenon

The phenomenon of electrostenolysis consists of the oxidation and reduction of a solution at the opposite surfaces of a high resistance membrane, which separates the two electrodes of an electrolytic cell Reduction of the solution takes place at the surface of the membrane facing the anode, oxidation at the surface facing the cathode, and the process occurs only if there is a steep potential gradient across the membrane   Some of the effects of the membrane are the same as those of a metallic conductor in the same position, in so far as ions are oxidized and reduced at the surfaces of both   The definition

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[1] *Cf* Blinks, L R , *J Gen Physiol* , 1929–30, 13, 361, 495   Osterhout, W J V , *Biol Rev* , 1931, 6, 369 (pp 397 ff)

[2] Lillie, R S , *J Gen Physiol* , 1935–36, 19, 109

[3] Cf Hill, A V , *Proc Roy Soc London, Series B*, 1936, 119, 305 (*cf* p 317)

stated above is more general than any so far given   Hitherto most
workers have been interested in the formation of metallic deposits
on the membrane surface, and have defined the phenomenon in terms
of these   However, as will be shown, these deposits are not its only
manifestations, and the above broader definition is needed

Although many workers have experimented with this phenomenon,
hitherto no one has applied quantitative methods to it,[4] consequently
such data as exist are of relatively little value   The present investiga
tion was undertaken in order to obtain quantitative information on the
efficiency of the process under varying conditions   Because of the
very small amounts of material transformed, the difficulty of obtaining
satisfactory results has been great, hence so far few data have been
obtained   However, a method has been developed which, on further
application, promises to give reliable quantitative results

EXPERIMENTAL

The first experimental requirement for a quantitative study of this phenomenon
is that the oxidation or reduction to be measured in the solution should take place
only at the membrane surface   This means that the experiments must be per
formed in a closed system in the absence of oxygen and hydrogen   that the solution
be free from any other impurities which would tend to oxidize or reduce it   and
finally that the electrodes of the cell be not too near the membrane and that
diffusion of solution from their neighborhood be minimized   Secondly, the
membrane must be inert in the sense that it must not oxidize or reduce the solu
tion without the passage of current   It should also be electrically stable $i e$,
its resistance should not vary excessively during the course of an experiment
Further it should not entrap products of the stenolysis which means that the
membrane must be thin   Since the potential gradient across it must be com
paratively high to produce any oxidation or reduction the conductivity of the
material used for making the membrane should be low with as high a break down
potential as possible.   Other general requirements for a suitable apparatus are
that there should be, adjacent to the membrane, a small volume of solution,
separable from the remainder of the solution in the cell which it is possible to

[4] There is a summary and bibliography in Freundlich H , Colloid and capillary
chemistry, New York E P Dutton 3rd edition, 1926, 271   Also see Grot
thuss T v , Ann Phys u Chem 1819, 61, 65   Recent papers which emphasize
especially the polar nature of the phenomenon are Lillie, R S , and Pond, S
E , Am J Physiol 1922 63, 415, Guastalla, L , and Urbain, G Compt rend
Acad 1934, 198, 1679, 1935, 201, 268   For a recent theoretical discussion cf
Söllner, K Z Elektrochem , 1929 36, 789

analyze   Provision must also be made for the easy and positive installation of the membrane in such a manner that, when in place, all of the current shall pass through, and none leak around it

The use of a potentiometric method of analysis was, from previous experience with colorimetric analysis, judged to be the best method for a determination of the amount of material transformed at the membrane   If, for example, in the ferrous-ferric oxidation, before and after a known quantity of current is passed, the potentials of the solution are measured against an inert platinum electrode, the change in ratio of reduced to oxidized ions can be calculated from the equation

$$E_h = E_0 - \frac{RT}{nF} \ln \frac{(\mathrm{Fe}^{++})}{(\mathrm{Fe}^{+++})}$$

If the volume and concentration of the solution are known, the number of moles of material so changed per unit current may be computed   The solution to be used as an indicator must be in a reduced state for two reasons   first, to make sure of the absence of oxygen, and secondly, to take advantage of the slope of the E_h vs (Reductant)/(Oxidant) curve, so that a small change in ratio may produce a large change in potential [5]

The final form of the apparatus is shown in Fig 1   This cell and the earlier cells were designed with a view to the investigation of compounds which would form no deposit on the membrane   The chamber B in this cell is equipped with three platinum wire electrodes, sealed into soft glass tubes, and contains also the terminus of a saturated calomel cell, connected to it by a ground joint and stop-cock, which can be filled with oxygen-free potassium chloride solution from the reservoir   One of the platinum electrodes is used, in conjunction with a similar one on the other side of the plate A, to measure the voltage drop across the membrane which is fastened on this plate   The drop of potential in the centimeter of solution which separates them is negligible in comparison   The solution in B is kept stirred, while the potential measurements are in progress, by moist nitrogen bubbled into it through a tube at the base of the chamber, attached to the nitrogen supply and pump line   The principal connection of this line is through the left-hand electrode, or cathode, chamber   The glass springs make allowance for slight differences in the position of the cell when it is set up, they allow the connections to be readily resealed, and the ground joint of the calomel cell to be put together with very little strain   The "square helix" used was found to be far more flexible than an ordinary helix   The cell is tightly clamped at the cathode chamber only and loosely supported elsewhere   If otherwise mounted, it was found that it was impossible to eliminate strains sufficiently to prevent its cracking   The large stop-cock, the core of which is shown enlarged (c), combines several simple cocks, thus facilitating the manipulation of the

[5] See Clark, W M , Studies on oxidation-reduction, I–X, *Bull Hyg Lab* , *U S P H S* , *No 151*, 1928

solution   The reducing flask is two flasks in one   the outer contains platinized asbestos and in this the reduction takes place, the inner one provided with a linen filter at the bottom, serves to store the solution out of contact with the platinized
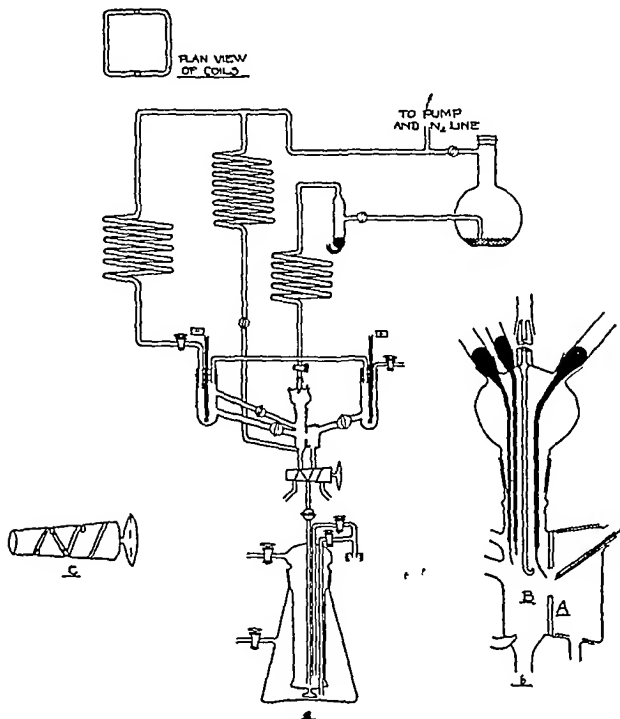
FIG 1

asbestos   To be able to increase the hydrogen pressure in the outer flask without blowing apart the joint it is sealed with Picëin cement   The flask is supported in position on a jack and is connected to the nitrogen and hydrogen lines with

flexible copper tubing (Bendix Aviation Corporation) sealed on to the glass tubes with de Khotinsky cement   This tubing was used in preference to rubber because of the noticeable diffusion of oxygen through the latter

For the measurement of the voltage drop across the membrane, a 40 volt potentiometer with 1/10 volt steps was used, the insertion of 80,000 ohms in the circuit minimized the possibility of error from electrolysis at these electrodes The current which passed through the cell was measured with a milliammeter, and the oxidation-reduction potential with a Leeds and Northrup student potentiometer   This circuit contains two galvanometers, one, a small desk type, for the rapid voltage drop measurements and for the rough adjustment of the other, more sensitive one, used for the determination of oxidation-reduction potentials

The most satisfactory membrane of several tried appeared to be that made from cellulose acetate   A 1 to 3 per cent solution in acetone was spread on mercury and allowed to dry very slowly   This gave a stable membrane of high resistance Its mean apparent thickness could be determined from the volume and concentration of the solution used, the area of the dry film, and the density of cellulose acetate   This type of membrane was used throughout these experiments   It was fastened to the cell with glyptal cement (General Electric Co)   This was very satisfactory inasmuch as it has excellent insulating properties and is entirely unaffected by the solution even after 10 days, provided, of course, it is allowed to dry thoroughly before use   Solutions of 0 1 molal ferric chloride and ferrous ammonium sulfate were used   A sample of the best grade of these salts obtainable was weighed out to 1 mg without further purification, for although impurities might influence the potential measured, this error would be virtually cancelled, as only the difference between two potentials is used in the calculations   The tanked hydrogen and nitrogen were effectively freed of oxygen, the former by passing it over hot platinized asbestos, the latter over hot copper   Both gases were moistened, to reduce evaporation of the solutions, by bubbling through oxygen-free water   The platinized asbestos used in the reducing flask was prepared by the ordinary method, and was washed with great care   The platinum electrodes in B were cleaned by electrolysis in strong hydrochloric acid, washed in water, then in dilute ammonium hydroxide, and finally in water again   They could also be removed and heated to white heat

The solution, in the outer part of the reducing flask, was boiled at reduced pressure to free it and the flask from oxygen, hydrogen was introduced and the process repeated several times   A pressure of about 15 cm of mercury was applied to both inner and outer containers, and the flask shaken mechanically until the reduction was complete   The pressure on the inner flask was released and the solution forced through the filter up into it, enough pressure being maintained in the outer flask to support the solution therein   Nitrogen was then passed through it for 3 or 4 hours to remove dissolved hydrogen   With the reducing flask in place under the cell, the system was freed of oxygen by alternately evacuating with an oil pump and filling it with pure nitrogen   The cell

was then filled with solution and allowed to stand overnight to soak the membrane and to be certain of the absence of oxygen (Its presence could be detected by a change of the potential of the solution) Saturated potassium chloride was run into the end of the calomel electrode, and while the solution was stirred, measurements of its oxidation reduction potential were made until the readings became constant After the stop-cocks were properly adjusted, current was passed between the two platinum coils in the electrode vessels, its direction being such that oxidation took place in B Readings were made, as nearly simultaneously as possible, of the current, the voltage drop across the membrane, the time and the temperature. Cotton plugs inserted in the bores of the two large stop-cocks which lead to the electrode vessels and which were necessarily open during the passage of the current, reduced diffusion from these vessels to the vicinity of the membrane After a known interval of time, the current was shut off, these same two stop-cocks closed the cock in the vent above B opened, and the solution therein stirred The oxidation reduction potential was then measured until the readings became constant the constant value serving for the final reading of the first experiment and the initial reading for the second, which was carried out immediately

RESULTS

Fig 2 shows the results of some rather inaccurate colorimetric experiments on methylene blue   The efficiency of the process in terms of equivalents oxidized per faraday passed, is plotted against the voltage drop per centimeter across the membrane   Each symbol corresponds to a series of experiments   The broken line was drawn without regard to the points designated as belonging to the 3–21 or 3–27 series It would not be wise to attempt to draw definite conclusions from these data because of their uncertainty   However, there does seem to be a minimum potential gradient necessary to oxidize the dye   This is in the neighborhood of 6700 volts per centimeter   It will also be noticed that most of the above mentioned points (of the 3–21 and 3–27 series) show a relatively high efficiency, in these runs ferrous iron was present in small quantities   If this greater efficiency is real, the iron seems to act as a catalyst for the oxidation   This is of special interest in view of the rôle played by iron as oxidation catalyst in living tissues where it (or other similar elements) is invariably found   The most important and most certain conclusion which may be drawn is that methylene blue is subject to stenolytic decomposition   No organic molecule has previously been shown to be affected by this process

It was found that not only was the leuco-methylene blue oxidized, but that the insoluble methylene white base was often deposited on the surface of the membrane facing the anode
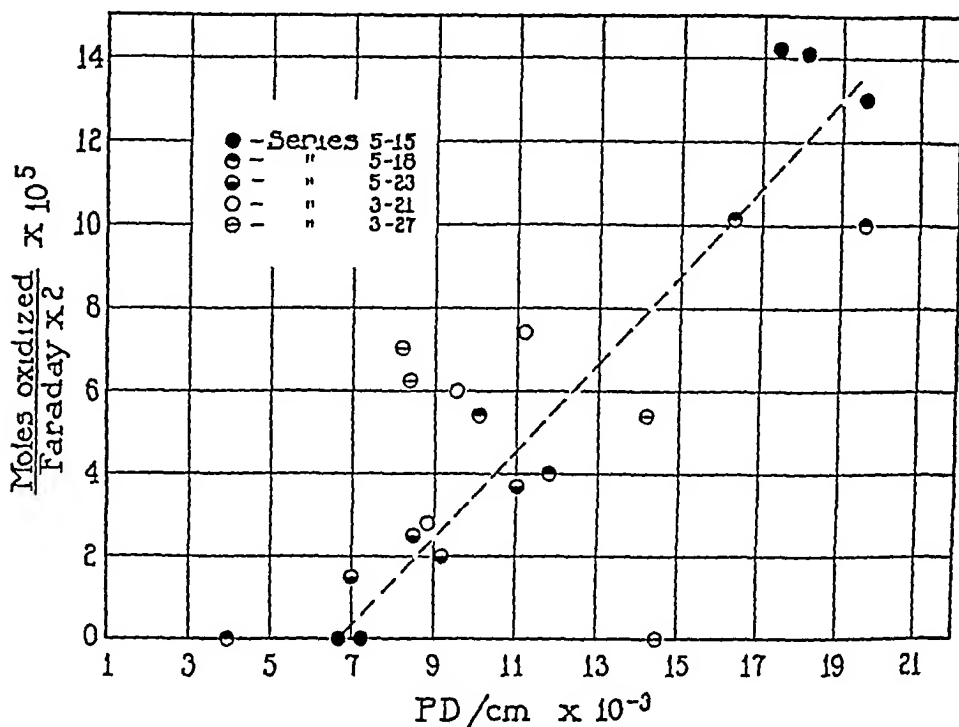
FIG 2

The results obtained by the use of the potentiometric method are all for the ferrous-ferric system   The number of moles of ferrous ion oxidized is given by the equation

$$E_h = E_0 - \frac{RT}{nF} \ln \frac{(Fe^{++})}{(Fe^{+++})}$$

E_h is the potential measured referred to the hydrogen electrode   (The voltage of the calomel cell used was taken as $+0\,2458$ at $25°C$) E_0 is the normal oxidation-reduction potential, taken as $+0\,7434$ at $25°C$[6] R is the gas constant, T the absolute temperature, n the number of electrons involved in the change from one state to the other,

[6] Popoff, S , and Kunz, A H , *J Am Chem Soc* , 1929, 51, 382

and F is the faraday constant   The number of moles of ferric ion present can be calculated by the following relation from the ratio (r) of the electrode equation, if the volume in cubic centimeters (v) of the solution in the chamber and its molal concentration (M) are known

$$\frac{m}{r+1} = \text{moles Fe}^{+++} \quad \text{where } m = \frac{vM}{1000}$$

Since E_A was measured before and after the passage of the current through the membrane, the initial and final number of moles of ferric ion could be calculated   The difference between these quantities is the number of moles of ferrous ions oxidized to ferric   The area under the time current curve was measured in order to evaluate the number of coulombs passed   The ratio of the number of moles of Fe^{++} oxidized, to this quantity, gives the efficiency   The potential drop across the membrane was measured directly, the mean apparent thickness of the latter was calculated from its area, the volume and concentration of the solution used to make it, and the density of cellulose acetate,—1 26 gm /cc   The data of a typical experiment are given in Table I t is the time, I, the current in milliamperes, P D is the potential drop across the membrane in volts, E_1 and E_2 are the potentials of the two electrodes used to measure the oxidation, T is the temperature in degrees Centigrade   The solution used was 0 1 M FeCl$_2$

In Table II the data obtained by this method are summarized, arranged in order of increasing voltage drop across the membrane The majority of the experiments are not represented in the table, for as many as fifteen were made in one series   When the results were examined, it was found that the later experiments of each series seemed to be far more efficient than the earlier ones, irrespective of the potential across the membrane   After the first few experiments the effect increased with the time elapsed after the series was started, and with the total current passed during the series   These latter quantities are closely related   The curve was flat for the first few hours, then rose very steeply, and finally started to level off   A rough calculation showed that it would take about 8 hours for a ferric ion to be carried from the anode to the membrane, this time is decreased by the agitation of solution caused by the turning of stop-cocks, diffusion,

etc Apparently then, transference of the ferric ion formed at the anode through the membrane into the chamber where the analysis was

TABLE I

Experiment Q_1 of Table II   Solution 0 1 M $FeCl_2$

t	I	P.D	E_1	E_2	T
8 33	—	—	+0 2402	+0 2402	23 8
8 41	—	—	0 2402	0 2402	23 7
8 50	—	—	0 2402	0 2402	23 9
8 57	—	—	+0 2402	+0 2402	23 9
9 00	17 6	1 7	—	—	—
9 03	17 3	2 4	—	—	24 0
9 05	17 15	2 7	—	—	—
9 07	17 1	2 8	—	—	—
9 10	17 05	2 8	—	—	24 0
9 15	17 0	2 8	—	—	—
9 20	17 0	2 7	—	—	24 0
9 25	17 0	2 7	—	—	—
9 30	16 9	2 7	—	—	24 1
9 35	16 8	2 7	—	—	—
9 40	16 8	2 7	—	—	24 0
9 45	16 8	2 7	—	—	—
9 50	16 8	2 7	—	—	24 1
9 55	16 8	2 7	—	—	—
10 00	16 75	2 7	—	—	—
10 04	—	—	+0 2443	+0 2440	24 2
10 10	—	—	0 2440	0 2438	24 2
10 15	—	—	0 2437	0 2436	24 1
10 21	—	—	0 2436	0 2435	24 1
10 30	—	—	0 2435	0 2435	24 1
10 40	—	—	+0 2435	+0 2435	24 2

Average potential drop	2 70 volts
Thickness of membrane	5 33 × 10⁻⁴ cm
Potential drop/cm	5 07 × 10³ v /cm
No moles oxidized	1 30 × 10⁻⁸
No faradays passed	6 34 × 10⁻⁴
No moles oxidized/faraday	2 05 × 10⁻⁵

made was the cause of the anomalously great efficiency observed after the first experiments After a time the ferric ions should enter the

chamber B through the membrane at the same rate they leave it, this approach to equilibrium is observed in the "efficiency" time curve referred to above   On the basis of this reasoning, all runs after the first three were discarded   At the start of a series, transference across the membrane could not cause important errors because the solution on both sides was the same   The effect (in the opposite direction) of diffusion through the membrane was likewise minimized by the selection of only the first runs, and the rejection of runs in which the potential did not become rapidly constant   Runs were also discarded when the potentials of the two oxidation reduction electrodes differed by

TABLE II

Experiment No.	No faradays	No m les oxidized	Moles oxidized / No faradays	ε_D/cm. × 10^{-3}	ε_D (Obs.)
P 1	4 44 × 10^{-4}	0	0	1 27	11 9
P 2	4 92	0	0	1 45	13 2
T 2	1 99	4 3 × 10^{-4}	2 16 × 10^{-4}	1 83	2 65
T 3	2 83	4 1	1 45	2 21	3 20
P 3	6 42	0	0	2 32	17 2
T 1	3 87	6 6	1 71	3 24	4 70
Q 1	6 34	13 0	2 05	5 07	2 70
Q 3	8 29	21 0	2 5	7 88	4 20
Q 2	8 44	15 0	1 8	8 08	4 30
U-4	2 24	3 0	2 3	13 3	19 3
U 3	1 36	3 0	2 2	15 8	22 7
U 2	1 64	4 0	2 4	16 4	23 8

more than 0 1 mv , usually it was not possible to read any difference between them on the student potentiometer   The greatest accuracy is realized only when the order of magnitude of the number of moles of ferrous ion oxidized is the same as the number of moles of ferric ion present   If this condition is not fulfilled, one, or even two decimal places will be lost when the difference is taken between the initial and final values of the latter   Another error is caused by the too large volume (20 cc) of the chamber where the analysis is made, however, if this is reduced too far, the errors of diffusion and transference become much more important   The errors relative to the determination of the number of faradays, the average potential drop,

and the thickness of the membrane are negligible compared to those mentioned above

The data of Table II are plotted in Fig 3 which gives the efficiency of the process in terms of moles oxidized per faraday passed, against the potential drop per centimeter across the membrane   It seems from the shape of the curve that there is a minimum potential gradient below which no oxidation occurs, this minimum falls at about 2150 v /cm   Lillie and Pond[4] estimated 2000 v /cm as the minimum at which they could effect any oxidation of ferrous ion at the surface of a rubber membrane   Considering the inaccuracies involved in the determination of the thickness of the membrane, this is excellent agreement   Whether the actual curve follows in detail the one in
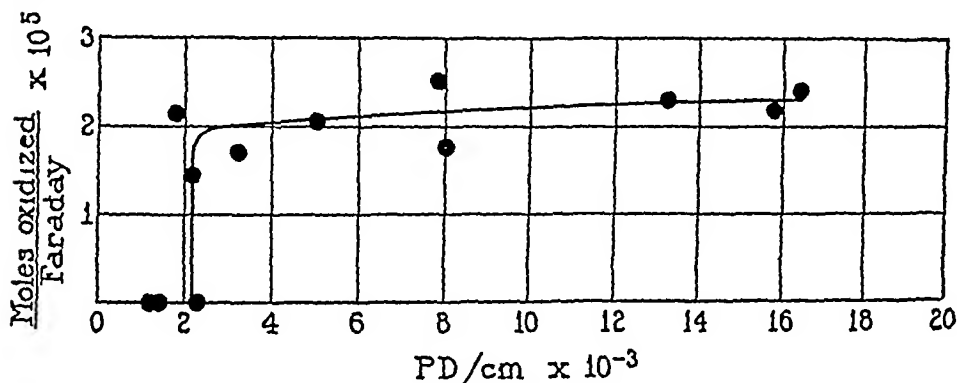
Fig 3

the figure must be determined by further experiment, for not enough data are available to make this curve certain in every respect   Although no data are presented, and those available are of doubtful accuracy, there is some evidence that the efficiency begins to fall off above about 17,000 v /cm possibly falling again to zero in the neighborhood of 22,000 v /cm

Any extensive theoretical treatment of this subject, based on data at present available, must necessarily belong largely in the realm of speculation   Nevertheless, a few suggestions of possible mechanisms concerned in this reaction seem not inappropriate   First, the possibility that electrostenolysis is due to capillary-electrical fields, as Coehn[7] suggested, seems remote, inasmuch as these fields become

[7] Coehn, A , Z Elektrochen , 1898, 4, 501, Z physik Chem , 1898, 25, 651

neglıgıble at the salt concentratıons used   In thıs connectıon ıt would be ınterestıng to ınvestıgate the change ın the effect wıth the concentratıon   It ıs not beyond the range of possıbılıty that an ınduced charge on the membrane, actıng ın conjunctıon wıth the relatıvely hıgh velocıty of the ıons (approxımately 1 3 cm /sec. at 2000 v /cm for ferrous ıon) whıch pass through the membrane pores, would serve to dıscharge some of them, by some such hypothesıs, the mınımum potentıal could be explaıned, and, ıf ıt exısts, probably also the maxımum potentıal   A study of the change of the effıcıency and of the possıbly more sıgnıfıcant quantıty, the mınımum stenolytıc potentıal, would show whether the membrane acts ın some specıal capacıty, or whether ıt merely serves to concentrate the ıonıc flow ınto several mınute pores ın whıch the stenolysıs ıs effected   On the basıs of the agreement between the mınımum stenolytıc potentıal found ın thıs ınvestıgatıon and that found by Lıllıe and Pond wıth a very dıfferent membrane, although thıs agreement may be purely coıncıdental, the latter hypothesıs seems the better   If the mınımum stenolytıc potentıal found for methylene blue (6700 v /cm) ıs approxımately correct, the dıfference between thıs value and that for ıron ındıcates a varıatıon of thıs quantıty wıth the ıon ınvolved, most probably a connectıon not only between the sıze of the ıon, but also ıts normal oxıdatıon reductıon potentıal

No purpose could be served by further speculatıon, sınce the maın object of thıs paper has been to present a method, wıth suffıcıent data secured by ıts use to demonstrate ıts feasıbılıty

SUMMARY

A quantıtatıve method for the ınvestıgatıon of electrostenolysıs has been developed   Electrostenolysıs ıs redefıned ın the lıght of the dıscovery that organıc molecules are subject to ıt   The experımental requırements for a quantıtatıve study are enumerated, and the apparatus and procedure descrıbed

It ıs found that wıth ferrous and ferrıc ıons and a cellulose acetate membrane, the potentıal drop across the membrane must be above about 2150 v /cm ın order to effect any oxıdatıon and reductıon   Wıth the present apparatus and condıtıons the ratıo of equıvalents oxıdızed or reduced to faradays passıng the membrane ıs low,— of the order of one to several thousand

CHANGES OF APPARENT IONIC MOBILITIES IN PROTOPLASM

II THE ACTION OF GUAIACOL AS AFFECTED BY pH

By W J V OSTERHOUT

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

Under the influence of guaiacol the P D of *Valonia* becomes more positive within 30 seconds[1] and the effect of the guaiacol ion appears to be much the same as that of the undissociated molecule   But after longer exposure (5 minutes or more) the cell seems to become sensitized to the guaiacol ion whose effect then appears to be much greater than that of the undissociated molecule

Fig 1 shows the P.D across the protoplasm of a cell (impaled on a capillary[1]) in sea water at pH 8 2   At the start it was slightly positive   On transferring to sea water at pH 6 4 there was practically no change in P.D [1]   On adding 0 01 M guaiacol (called for convenience HG) the P D became about 75 mv positive (the pH was kept[3] at 6 4) When the pH was raised[3] to 9 6 there was little change in P D although the concentration of the guaiacol ion (called for convenience G^-) was thereby raised[4] from about 0 000007 M to about 0 0043 M

In Fig 1 we see that at pH 9 6 there is a tendency for the curve to rise   This is sometimes absent but in some cases it is much more pronounced than in Fig 1   (After a longer exposure to HG we see the opposite effect since a rise in pH then causes the curve to fall as in Fig 2)

[1] Osterhout W J V *J Gen Physiol* 1936-37 **20**, 13

   Positive means that the positive current tends to flow from the vacuole across the protoplasm to the sea water   The cells are usually slightly negative in the sea water but a slight positivity such as we see here seems to be without significance

[3] This is done by adding NaOH   The technique and material were the same as in the previous paper [1]

[4] See page 689
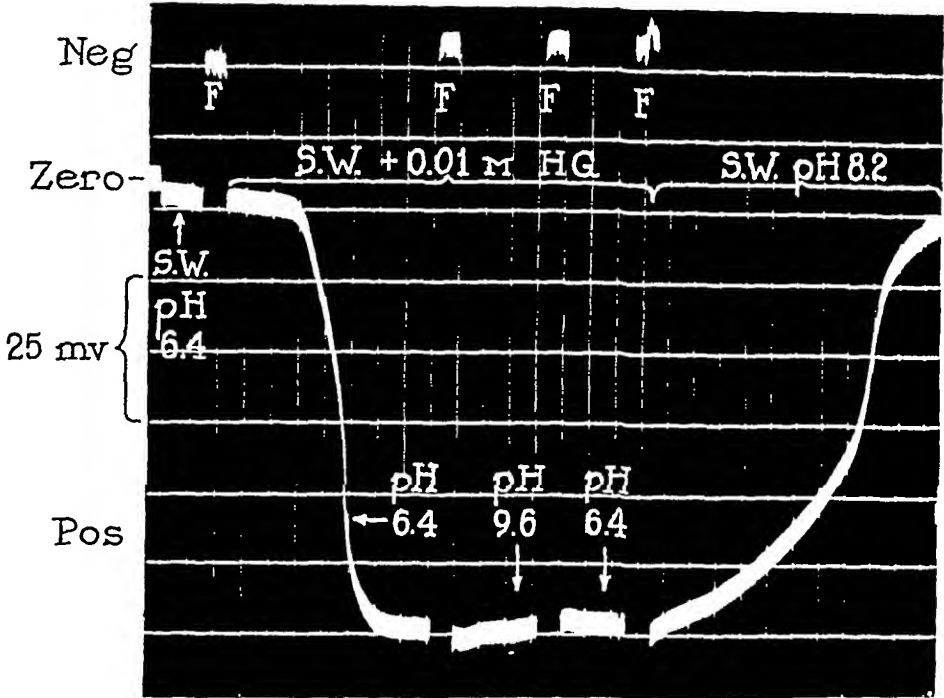
FIG 1
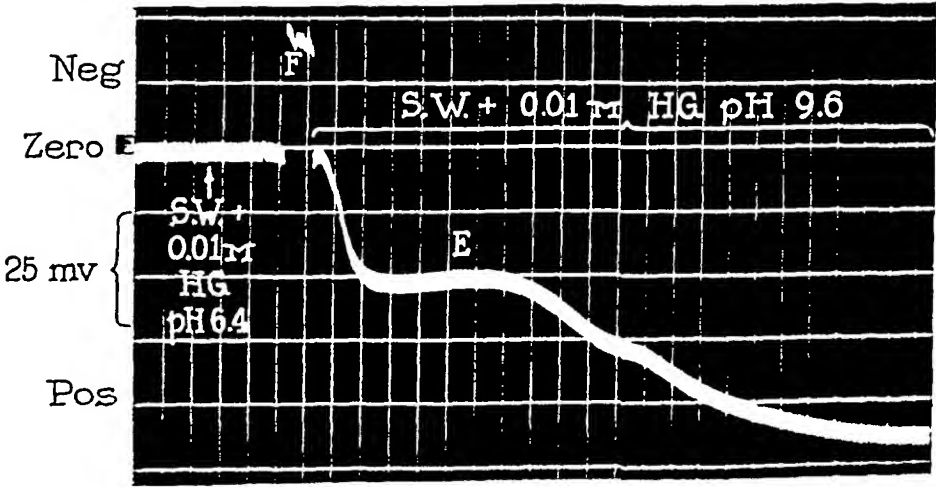
FIG 2

FIG 1 Photographic record showing effect of replacing sea water at pH 6 4 by sea water + 0 01 M HG at pH 6 4 (where the concentration of the guiaicol ion is negligible) In sea water the cell was slightly positive When the cell is lifted out of the sea water the curve jumped up to F, the free grid of the vacuum

Fig 1 shows that when the cell is replaced in sea water (at pH 8 2) it recovers its original P D   This also happens when the pH of the sea water is 6 4   If the cell is not replaced in sea water the curve slowly rises and the P D may reach the original value [s] for convenience we speak of this as "recovery" but without implying that the cell returns to its original state (this has been discussed in a previous paper[1])

Let us now see what happens after a longer exposure to HG (such observations were first made in 1931 by L R Blinks, who kindly tested the effect of guaiacol at the request of the writer)   Fig 2 shows a typical result   The cell, which had a P D at the start of 3 mv negative, was placed in HG 0 01 M at pH 6 4 for 300 seconds during which time the curve fell to 55 mv positive and then rose nearly to zero where it remained fairly steady   This is the condition shown in the figure at the start   The pH was then raised to 9 6 and the curve began to fall after approximately 2 seconds, which is hardly long enough to be regarded as a latent period

tube amplifier, when it was placed in sea water + 0 01 M HG it jumped back again and after a latent period[1] of about 14 seconds began to descend   After it reached its lowest level the pH was raised to 9 6 which produced very little change Changing back to pH 6 4 left the P.D practically unaltered

Time marks 5 seconds apart   Temperature 22 C

The positive drop in this case was larger than the average reported in the previous paper [1]   The amount was quite variable in this lot of cells

Fig 2   Photographic record showing the effect of a rise in pH (and hence in the concentration of the guaiacol ion) on a cell of *Valonia* which had been previously exposed to sea water + 0 01 M HG for 300 seconds during which time the P.D became about 50 mv more positive than at the start and then returned to the original value

At the start of the curve shown in the figure the cell was in sea water at pH 6 4 containing 0 01 M guaiacol   When it was lifted out the curve jumped to the free grid (F) of the vacuum tube amplifier   When it was again placed in the same solution with its pH raised to 9 6 the curve began to descend after about 2 seconds

The fall in the first part of the curve (about 30 mv) may be attributed to the effect of the guaiacol ion on the outer protoplasmic surface, the remainder (after the letter L, amounting to 34 mv) may be due to other causes

Time marks 5 seconds apart   Temperature 20°C

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[s] This happens in sea water + 0 01 M HG at pH 6 4 or 9 6   The "recovery" in this case is much slower than in sea water and is much more apt to be incomplete
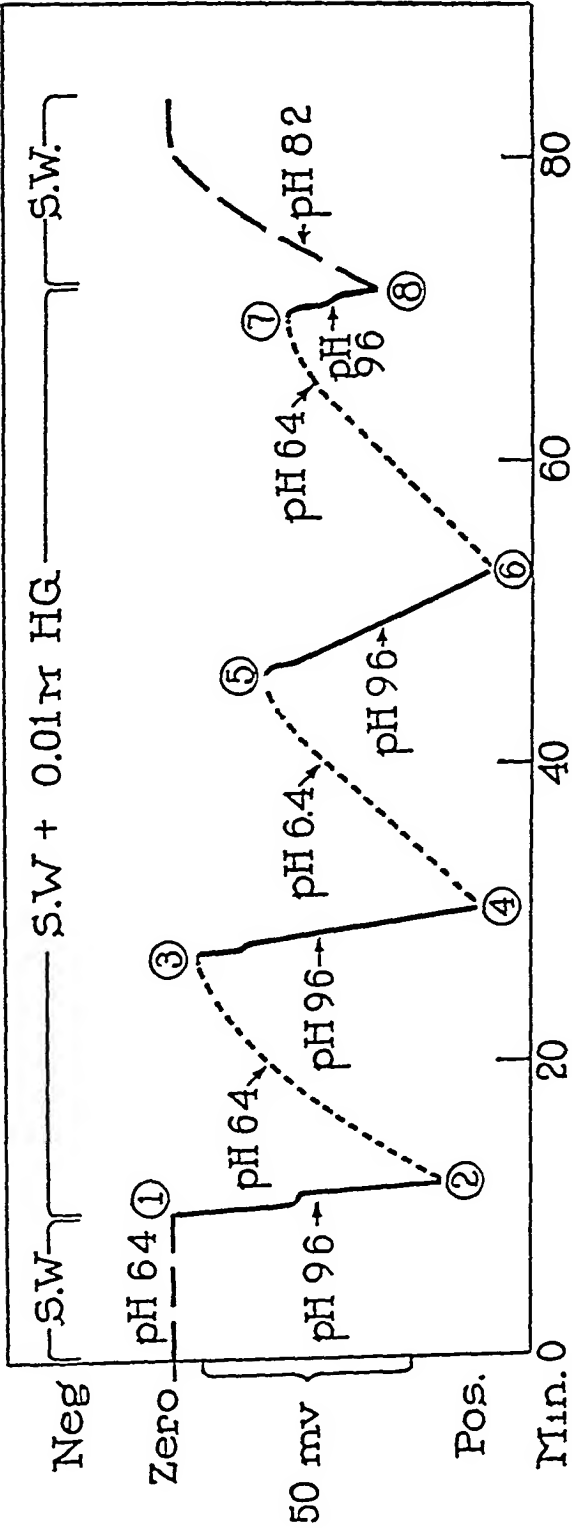
FIG 3 Reproduces approximately the curve shown in Fig 2 with a much smaller time scale and shows the subsequent behavior of the cell   At 1 the cell was transferred from sea water containing 0 01 м guaiacol at pH 6 4 to the same solution with the pH raised to 9 6   At 2 the pH was lowered to 6 4 and this alternation continued throughout the curve until at 8 the cell was placed in sea water   Temperature 20°C

When the pH is lowered to 6 4 the curve rises   By changing the pH several times we obtain the effects shown in Fig 3

It may be added that in these experiments[6] there was as a rule no evidence of permanent injury [1]   The impaled cells lived on for several days after the experiment

DISCUSSION

The positive drop in Fig 2 coincides with the increase in the concentration of the guaiacol ion (G^-) from about 0 000007 M to about 0 00429 M due to the change in pH

The concentration of G^- was calculated as follows   According to Shedlovsky and Uhlig[7] the pK of HG at 25°C is 10 1   Subtracting 0 375 for the ionic strength of sea water[8] gives pK = 9 725   Accordingly[9] at pH 6 4 the degree of dissociation is 0 07 per cent and if the concentration of HG is 0 01 M the concentration of G^- is 0 000007 M   At pH 9 6 the degree of dissociation is 42 9 per cent and the concentration of G^- is 0 00429 M

During the first part of its course the form of the curve in Fig 2 is just what we should expect if G^- had a high mobility and were brought suddenly in contact with the outer protoplasmic surface, i e there is little or no latent period and the curve is steep at first and gradually flattens out just as when an ion (e g K^+)[10] which has a high mobility is applied to *Valonia*[11] or to *Nitella* [12]

After becoming horizontal[13] the curve begins to descend again   It

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[6] If any sign of injury appeared the experiment was rejected

[7] Shedlovsky T , and Uhlig, H H , *J Gen Physiol* , 1933–34, **17**, 567

[8] Jacques A. G   *J Gen Physiol* , 1935–36, **19**, 397

[9] This is obtained by means of the formula pH = pK + log $\dfrac{a}{1-a}$, where a is the degree of dissociation   Cf Clark, W M , The determination of hydrogen ions, Baltimore The Williams and Wilkins Company 3rd edition 1928, pp 15 and 677

[10] In the case of K^+ the curve rises instead of falling because of the opposite charge on the ion

[11] Damon, E B , *J Gen Physiol* , 1932–33, **16**, 375   Osterhout, W J V , *J Gen Physiol* 1936–37 **20**, 13 (Figs 8 and 9)

[12] Osterhout W J V *J Gen Physiol* , 1929–30 **13**, 715 (Fig 2)

[13] In some cases the horizontal part of the curve is very short or in place of the horizontal part of the curve there is a gradual descent as if the second process had begun before the first was over   In a few cases there was no indication of two

seems possible that this second descent is due to an alteration of the protoplasm by G^- somewhat analogous to that produced by undissociated HG [1]

If we adopt this as a working hypothesis for the time being and regard the first descent of the curve as the result of a change in diffusion potential due to the high mobility of G^- we can calculate the apparent mobility of G^- by means of Henderson's equation

This may be written (for 20°C)

$$P D = 58 \frac{(U_I - V_I) - (U_{II} - V_{II})}{(U_I + V_I) - (U_{II} + V_{II})} \log \frac{U_I + V_I}{U_{II} + V_{II}}$$

Since the concentration of H^+ is very small it may be neglected and then we have $U_I = U_{II}$ so that the equation becomes

$$P D = 58 \log \frac{U_I + V_I}{U_{II} + V_{II}}$$

where

$$U_I = U_{II} = C_{Na} U_{Na} + C_K U_K$$
$$= (0\,488)(0\,2) + (0\,012)(20)$$
$$= 0\,388$$

We also have[14] (putting for convenience $V_{Cl} = 1$) at pH 9 6

$$V_I = C_{Cl} V_{Cl} + C_G V_G$$
$$= (0\,5)(1) + 0\,00429\,V_G$$

and at pH 6 4

$$V_{II} = (0\,5)(1) + 0\,000007\,V_G$$

When a rise of pH from 6 4 to 9 6 causes a change of P D of 30 mv (as in the first descent of the curve in Fig 2) we have

$$30 = 58 \log \frac{0\,338 + 0\,5 + 0\,00429\,V_G}{0\,338 + 0\,5 + 0\,000007\,V_G}$$

whence $V_G = 450$

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steps but the curve fell somewhat as in Fig 1 (perhaps because the exposure was too short)

Occasionally after the first descent of the curve we find instead of a short horizontal stretch a rise (as though recovery had begun) followed by a fall of the curve

[14] Regarding the numerical values see Damon, E B , J Gen Physiol , 1932–33, 16, 375

We thus arrive at a very large value (450) for the apparent mobility of G^- (when that of Cl^- is taken as unity) which is the more surprising since it does not appear to exist after a brief exposure to HG   At that time G^- appears to have much the same mobility as Cl^- as shown by the fact that increasing the concentration of G^- does not increase the positive drop[15] (Fig 1)   (It seems rather to have somewhat the oppo site tendency, at least in some cases)

This raises the question whether the effect of pH on the first part of the positive drop in Fig 2 may be due to something else than a change in the apparent mobility of G^- (as is probably the case with the second part of the drop in the curve)   It might be suggested, for example, that injury is responsible but injury would be expected to carry the P D toward zero rather than away from it

If we adopt as a working hypothesis the suggestion that exposure to HG increases the apparent mobility of G^- in the protoplasmic sur face we may ask how this comes about   As stated in a former paper[1] such great changes can hardly be due to alterations in partition coeffi cients or in formation of compounds in the protoplasm   It is more plausible to assume that they are the result of the formation of charged complexes

In conclusion it seems desirable to say something about "recovery " Recovery at pH 6 4 in sea water + HG, after a brief exposure, has been attributed[1] to the penetration of undissociated HG producing on the inner protoplasmic surface much the same effect as on the outer but opposite in sign, so that the P D returns toward its normal value [16]

After the cell has been sensitized by a long exposure to HG and the concentration of G^- is then suddenly raised the change of P D occurs so quickly that we may ascribe the first drop in the curve to a change in diffusion potential at the outer non aqueous protoplasmic surface This will tend to disappear as G^- penetrates and lowers the concentra tion gradient across the non aqueous layer at the protoplasmic surface In consequence the curve would tend to rise and produce recovery

In addition to this phenomenon we have the positive drop following

[15] It was stated in a former paper[1] that the positive drop was 28 0 mv at pH 6 4, and 26 2 at pH 8 2 (see Table I, p 16)

[16] Of course if the pH in the protoplasm is higher than 6 4 there would be a corresponding change in the dissociation of HG

the horizontal stretch of the curve in Fig 2   This may perhaps be due to alteration of the non-aqueous surface layer which brings about a change in the diffusion potential set up by organic ions in the aqueous layer of the protoplasm as they diffuse outward through the outer surface   When G^- reaches the inner protoplasmic surface and alters it there may be a corresponding change in the diffusion potential which will be opposite in sign because in this layer the organic ions are diffusing inward [1]   This would tend to produce recovery

These are merely suggestions which must await further tests before any definite conclusion can be reached   The situation is complicated by other variables   For example we should expect ions to penetrate more slowly than undissociated molecules,[17] but on the other hand CO_2 would pass out rapidly when the pH of the sea water was raised and this would raise the pH of the protoplasm and consequently increase the concentration of G^- in the protoplasm   This might change the P D since the apparent mobility of G^- may not be the same in the inner and outer protoplasmic surfaces [18]   Moreover the concentration gradient of G^- across the two protoplasmic surfaces would not be affected in the same way in both cases

It must be remembered that during the exposure to HG the apparent mobility[1] of K^+ and of Na^+, as well as that of G^-, appears to change so that a complicated set of variables is in play

In a former paper[1] it was found that after a brief exposure recovery in sea water $+ HG$ was slower at pH 8 2 than at 6 4 but in the present experiments (with longer exposure) it was sometimes slower and sometimes more rapid at 9 6 than at 6 4   After an exposure of 5 minutes it was on the whole much slower at 9 6 and the degree of recovery was highly variable

It may be added that when the P D has become normal after recovery in sea water $+ 0 01$ M HG at pH 6 4 or 9 6 (after long exposure) a change to normal sea water at pH 8 2 (containing no HG) usually has little or no effect on the P D   This may mean that the HG and G^- come out of the cell in such fashion as to affect both protoplasmic surfaces in much the same manner

Likewise after a brief exposure to HG recovery at pH 9 6 may bring

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[17] Osterhout, W J V , *Ergebn Physiol* , 1933, 35, 967, *Bot Rev* , 1936, 2, 283
[18] Damon, E B , *J Gen Physiol* , 1931–32, 15, 525

the curve back to normal and it is then found that lowering the pH to 6 4 does not raise it above normal

SUMMARY

The normal P D across the protoplasm of *Valonia macrophysa* is about 10 mv negative (inwardly directed)   On adding 0 01 M guaiacol to the sea water the P D becomes positive and then slowly returns approximately to the normal value

In many cases this behavior is not much affected by raising the pH and so increasing the concentration of the guaiacol ion but in other cases such an increase makes the P D somewhat more negative

But if we wait until the exposure to guaiacol has lasted 5 minutes (and the P.D has returned to its normal value) before we raise the pH, the result is very different   The cell then behaves as though it had been sensitized to the action of the guaiacol ion which appears to be far more effective than undissociated guaiacol in making the P D more positive   This may be due in part to the high apparent mobility of the guaiacol ion and in part to alterations which it produces in the protoplasm (such alterations increase the P D across the protoplasm whereas ordinary injury would be expected to lower it and the cells live on after this treatment and show no signs of injury)

This action of the guaiacol ion is in marked contrast to the behavior of other anions whose effect resembles that of Cl−

POLARIZATION STUDIES IN COLLODION MEMBRANES AND IN SYNTHETIC PROTEIN-LIPOID MEMBRANES*

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While investigating the polarization of brain tissue (1) adequate tissue models became desirable Some experiments along this line have been published before (2) with the method used for measuring the polarization

The method consisted briefly in measuring the conductivity of a membrane at various frequencies (560–5000 cycles) For this purpose the membrane was inserted in a three cell apparatus which was filled with a salt solution In case of polarization of the membrane there is a difference between the conductivity at high and at low frequency This difference expressed in percentage of the conductivity at low frequency (Δ) has been used as a measure of polarization The determinations of Δ require exact minimum positions on the bridge These can be much more quickly determined than the exact capacity necessary for absolute silence Therefore when studying experimental changes on living brain the former method seemed preferable To make direct comparisons possible the same method has been used here A mica decade condenser was used to determine the parallel capacities necessary to obtain a distinct minimum on the bridge

The experiments reported here deal mainly with three problems methods of preparing polarizable collodion membranes of low resistance and some of their properties, relations between polarization and permeability, methods of building a synthetic protein lipoid membrane which shows polarization, and its reaction to different substances

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* Parts of this paper have been presented before the meeting of the Federation of American Societies for Experimental Biology, at Washington, D C, March 26, 1936

I

It has been reported previously (2) that addition of lipoids to collodion causes polarizability in membranes, while membranes made under similar conditions from pure collodion are not polarizable No explanation has been offered up to this time, but at least three possible interpretations have commanded consideration specific effects of

TABLE I*

Influence of Holes on the Polarization of Lecithin-Collodion Membranes (I) and Pure Collodion (II) Membranes

No of holes	Lecithin-collodion					Collodion		
	K_{560} 10^{-4}	C	K_{5000} 10^{-4}	C	Δ	K_{560} 10^{-4}	K_{5000} 10^{-4}	Δ
					per cent			*per cent*
0	4 50	0 066	8 28	0 014	84	3 62	7 94	119 3
1	5 21	0 05	8 31	0 013	59 5	—	—	—
2	5 87	0 036	8 59	0 010	46 4	7 62	11 28	48 5
3	6 85	0 023	8 80	0 008	28 4	—	—	—
4	7 22	0 029	9 18	0 010	27 1	13 80	16 20	17 7
5	7 46	0 027	9 16	0 011	22 8	17 38	19 22	12 9
6	7 70	0 027	9 42	0 009	22 3	21 52	23 04	7 2
7	7 83	0 025	9 44	0 008	20 6	25 24	26 52	5
12	—	—	—		—	30 76	32 22	4 8
29	—	0 015	—	0 005	12 0	42 12	42 84	1 7
No membrane	—	—	—		—	90 96	90 96	0

* In all the tables, K = specific conductivity,
Indices 560 and 5000 = frequencies in cycles,

$$\Delta = \frac{K_{5000} - K_{560}}{K_{560}} \, 100,$$

C = capacity in μF

lipoids, changes in the degree of dispersion, and displacement of the solvent Therefore, the following experiments were undertaken

The specific action of lecithin for inducing polarizability can be interpreted as due to a formation of a continuous thin layer with high polarizability, as recently suggested by Bungenberg and Bonner (3) In order to test this assumption, a wire of 0 012 inch diameter was used to punch holes consecutively into a polarizable lipoid-collodion membrane If the polarizability is due to the intactness of the lecithin

film, an impairment of the film must destroy the polarizability   The results of such a series of experiments are summarized in Table I The figures indicate that interruptions of the continuity of the membrane only partly destroy the polarization of a lecithin collodion membrane   The decrease is marked after the first 3 holes   Nevertheless, the decrease does not run parallel to the number of holes   In fact, from the 5 to 7th hole the polarizability remains nearly stable and 22 more holes are required to reduce it again by half   The total area of the holes amounts to about 16 sq mm , while the surface of

TABLE II

Influence of Various Substances on the Polarizability of Collodion Membranes

Drying time	Material of membranes	$K_{in} 10^{-4}$	C	$K_{ex} 10^{-7}$	C	Δ
min						*per cent*
60	Pure collodion (Merck)	17 20	0 005	17 20	0 001	0
	Collodion Merck 2 per cent egg lecithin	11 7	0 025	12 4	0 005	14 2
	U S P 2 per cent mastix	23 0	0 005	23 1	<0 001	0
	Merck 5 per cent olive oil	8 68	0 012	9 24	0 009	6 2
75	Pure collodion U S P	22 8	<0 001	22 8	<0 001	0
	U.S.P 2 per cent gum benzoin	5 65	0 080	10 90	0 025	92 9
	U S P , 2 per cent mastix	1 54	0 020	1 58	0 010	2 9
90	Pure collodion U S P	20 4	<0 001	20 4	<0 001	0
	Collodion U S P 2 per cent camphor	6 27	<0 001	6 27	<0 001	0
'	U S P 1 per cent cholesterin	23 0	0 010	23 0	0 010	0
	U S P 2 per cent mastix	1 39	0 027	1 77	0 010	27 6

the membrane is about 452 sq mm   In view of these results it does not seem probable that the entire polarization of the lipoid collodion membrane depends upon the existence of a continuous lecithin film This opinion gains additional support through the fact that a polarizable membrane of pure collodion shows almost a similar behavior when submitted to the same treatment

In view of a former observation (2) and of the recent investigations of Fricke and Curtis (4), changes in the degree of dispersion of the membrane colloids had to be considered as possible causes of polariz

ability   No direct proof to the contrary can be given, but there are some facts that make this assumption improbable, at least, for the range of frequencies used

It was shown before (2) that it was not possible to produce polarizability by adding phosphatides to gelatin membranes   Neither were attempts successful to induce polarizability in collodion membranes by introducing other colloids, e g proteins   Finally it could be shown that lecithin sol containing the same amount of lipoid as the polarizable lecithin-collodion membrane, when placed in the middle cell of a three cell apparatus between two parchment membranes, did not show differences in conductivity when measured at different frequencies of the alternating current (Table VI A)

Experiments were begun in order to determine whether the action of the lipoids in rendering collodion polarizable was due to their action on one of the solvents of the collodion   Alcohol and ether are contained in both Merck collodion and U S P collodion   Since cephalin effects the polarizability of membranes quantitatively in the same way as lecithin and since it is insoluble in alcohol, its effect upon the ether was considered   The permeability of collodion membranes depends largely upon the ratio between ether and alcohol (5) and therefore, experiments were started in order to ascertain if additions of ether-soluble, non-volatile substances would act on the polarizability in the same way as lipoids (Table II)

The results summarized in Table II show that substances of different chemical character, such as olive oil, mastix, and gum benzoin when added to collodion are able to induce polarizability   Camphor and cholesterin which separate from solution when the collodion membranes solidify do not have this effect, or only to a very small degree These results seem to support the view that the influence of the addition of lipoids to collodion is upon the solvent

Through the information gained by these experiments the preparation of membranes of low resistance and marked polarizability is greatly facilitated   Nevertheless, this introduction of other substances into the collodion complicates the understanding of the mechanism underlying polarizability

Experiments were therefore started with the purpose of intercepting an intermediate between the wet and the dry stages in the preparation

of collodion membranes Wilbrandt (6) has given evidence that there is a steady transition from highly permeable wet to completely dried membranes The dry collodion membranes which have been

TABLE III

Effects of Drying under Pressure on Resistance and Polarizability of Collodion Membranes

(Apparatus filled with 0 02 N KCl, capacitance = 0 4 resistance of apparatus without membranes = 165 ohm = R_0 C_{560} = 0 015, C_{5000} <0 001)

Total time	Dried only		Δ	Dried for 1 hr and pressed for rest of time		Δ
	K	$R-R_0$		K	$R-R_0$	
			per cent			*per cent*
1 hr 30 min	At 560 cycles 1 90 10^{-3} C <0 001 At 5000 cycles 1 92 10^{-1} C <0 001	43	0	At 560 cycles 1 28 10^{-1} C = 0 014 At 5000 cycles 1.35 10^{-1} C = 0 007	132	5 8
1 hr 35 min	At 560 cycles 1 10 10^{-4} C = 0 020 At 5000 cycles 1 74 10^{-4} C = 0 010	22 89	59	—	—	—
1 hr 45 min.	Not measurable on account of high resistance			At 560 cycles 6.36 10^{-4} C = 0 014 At 5000 cycles 7 00 10^{-4} C = 0 007	407	10 1
2 hrs				At 560 cycles 4 04 10^{-4} C = 0 100 At 5000 cycles 6 04 10^{-4} C = 0 020	498	49 6

thoroughly investigated by Michaelis (7) and Northrop (8) are not adapted to the kind of investigations intended Although it was possible to duplicate the results found by Rein (9) with membranes

made according to Michaelis by directly measuring the membrane potential, the conductivity method which is successful in tissue measurements failed   This failure is probably due to the high resistance of the membrane   Michaelis (10) using the data of Fricke (11) and McClendon (12) came to the conclusion that the membranes of red blood cells have little permeability   However, the aim of this investigation was only to find models comparable to brain tissue in polarizability   After several attempts the following method was used   10 cc of collodion were allowed to fill a glass dish of 39 8 sq cm   After 60 minutes drying at room temperature the adherent membrane

TABLE IV

Influence of Electrolytes on Polarization

Electrolytes isophoretic with 0 02 N KCl	Non polarizable membrane			Polarizable membrane		
	$\frac{K}{C}\}$ 560	$\frac{K}{C}\}$ 5000	Δ	$\frac{K}{C}\}$ 560	$\frac{K}{C}\}$ 5000	Δ
			per cent			*per cent*
KCl	$2\ 82\ 10^{-2}$ $C = 0\ 001$	$2\ 82\ 10^{-3}$ $C = 0\ 001$	0	$1\ 75\ 10^{-3}$ $C = 0\ 050$	$2\ 01\ 10^{-3}$ $C = 0\ 015$	15 0
HCl	$2\ 91\ 10^{-3}$ $C = 0\ 01$	$2\ 91\ 10^{-3}$ $C = 0\ 001$	0	$1\ 74\ 10^{-3}$ $C = 0\ 014$	$2\ 02\ 10^{-3}$ $C = 0\ 006$	16 4
MgCl₂	$2\ 82\ 10^{-3}$ $C < 0\ 001$	$2\ 82\ 10^{-3}$ $C < 0\ 001$	0	$8\ 24\ 10^{-4}$ $C = 0\ 054$	$1\ 0S\ 10^{-3}$ $C = 0\ 013$	29 9
La(NO)₃	$1\ 13\ 10^{-3}$ $C < 0\ 001$	$1\ 13\ 10^{-3}$ $C < 0\ 001$	0	$5\ 54\ 10^{-4}$ $C = 0\ 04$	$7\ 4S\ 10^{-4}$ $C = 0\ 008$	35 1

was detached from its glass support and pressed for varying intervals of time between sheets of filter paper under a pressure of 50 gm /sq cm   The results are summarized in Table III

U S P collodion and Merck collodion behaved in practically the same way   The results show that the membranes made as described above are of moderate resistance and high polarizability   Furthermore, the superiority of this method over that of complete drying is evident   These "medium" dry collodion membranes show towards electrolytes many of the particular features of dry collodion membranes and living tissue   Their polarizability is increased when in equilibrium with isophoretic salt solutions of bi- and trivalent cations

or with HCl, but is decreased by isophoretic NaOH, collodion membranes containing the same amount of collodion but showing no polarizability are apparently uninfluenced by the higher valency of the ions or by the acidity of the solution (Table IV)

II

As the resistances of the polarizable membranes were in every comparable instance higher than the resistances of non polarizable membranes, it was natural to assume that they were less permeable for electrolytes than the non polarizable membranes   Under this assumption polarizability has previously (1–2) been used as a measure

TABLE V

Permeability and Polarization

Time	1 per cent lecithin-collodion membrane $\Delta = 0$	4 per cent lecithin-collodion membrane $\Delta = 29\ 5$
hrs		
0	1 11 10^{-6} reciprocal ohm	6 4 10^{-6} reciprocal ohm
1	8 40 10^{-6} "	1 33 10^{-6} "
2	1 98 10^{-4} " "	2 11 10^{-6}
4	2 24 10^{-4} " "	2 20 10^{-6} "
22 5	8 00 10^{-4} "	4 80 10^{-6} "
48	11 4 10^{-4} "	7 90 10^{-6} "

of permeability   In order to ascertain this fact in a quantitative way, the following experiments were started

Two collodion membranes were made in exactly the same way, both containing the same amount of collodion, but different quantities of egg lecithin, namely the first 1 per cent and the second 4 per cent. The respective resistances were 480Ω and 635Ω when measured in 0 02 N KCl   After a similar exposure to CaCl₂ Δ was 0 and 29 5 per cent respectively   Each of the membranes was then inserted into a three cell apparatus   On one side of the membrane there was 0 02 N KCl and the compartment on the other side of the membrane was filled with distilled water   At frequent intervals the electric conductivity of samples of the water were measured   The results of this experiment are summarized in Table V, indicating that the perme-

ability of the membrane showing polarization was approximately only one-fifteenth of that of the non-polarizable membrane

Another clue as to a relationship between permeability and polarization can be found in the behavior of a single membrane (Table VI), upon ageing

III

For the studies of some physicochemical problems the collodion membranes described above proved to be adequate tissue models But their shortcomings became manifest when the influence of biologically active substances was investigated   Therefore, the attempt was made to build up polarizable membranes in which the reactive

TABLE VI

Ageing of a 4 Per Cent Lecithin-Collodion Membrane

Time	Conductivity apparatus filled with 0 02 N KCl				Resistance of membrane	Δ
	K_{in} 10⁻¹	C	K_{100} 10⁻¹	C		
days					*ohms*	*per cent*
0	4 99	0 06	6 46	0 025	636 6	29 5
3	7 32	0 025	7 95	0 011	381 5	8 6
6	10 91	0 017	11 28	0 008	202 6	3 4

parts consisted solely of substances occurring in animal and human tissues

No such investigations have so far been successful   Fricke and Curtis (4) could not find polarization in a solution of gelatin, and Abramson and Gray (13) were unable to detect changes in permeability in collodion membranes containing up to 50 per cent lecithin

Therefore, the problem was attacked from different angles   In the first series of investigations collodion was used as a supporting medium, and different proteins (serum, egg albumin) were introduced into it in the same way as in the membranes used in electrodialysis by Ettisch and Ewig (14)   By thoroughly shaking (2 hours on a shaking machine) a certain homogeneity was reached   The membranes were dried in such a way that pure collodion under similar treatment would not yield a polarizable membrane   No trace of polarizability could be

TABLE VI A

Unsuccessful Attempts to Render Membranes Polarizable by Introducing Biological Colloids or to Build Membranes with These Colloids

Supporting material	Introduced material	K_{560} 10⁻²	C	K_{5000} 10⁻²	C	Δ
Collodion	Blood albumin 0 2 gm shaken in 10 ml collodion dried for 60 min	1 94	<0 001	1 94	<0 001	0
'	As above, treated for 10 min with 0 02 N CaCl₂	2 06	<0 001	2 06	<0 001	0
'	2 per cent blood albumin in collodion mechanically shaken for 60 min , in 0 02 N KCl	1 46	<0 001	1 46	<0 001	0
	As above in 0 01 N HCl	1 78	0 004	1 78	<0 001	0
	As above in 0 01 N NaOH	2 69	0 005	2 69	<0 001	0
'	As above electrodialyzed for 40 min	2 31	<0 001	2 31	<0 001	0
	Egg albumin 2 per cent in collodion mechanically shaken for 3 hrs dried for 60 min	1 71	<0 001	1 71	<0 001	0
30 per cent gelatin	2 per cent heat denatured egg albumin	2 03	<0 001	2 03	<0 001	0
As above	As above after electrodialysis	3 55	<0 001	3 55	<0 001	0
Solid gelatin	Treated 24 hrs with formaldehyde pressed 0 02 N KCl + formaldehyde	2 18	<0 001	2 18	<0 001	0
3 gm gelatin	Treated with 36 per cent formaldehyde for 2 hrs 0 02 N KCl + formaldehyde	1 41	<0 001	1 41	<0 001	0
Filter paper	1 gm casein in 5 ml 0 1 N NaOH spread 3 days in 10 per cent formaldehyde	2 00	0 010	2 00	0 001	0
'	As above in 0 012 N HCl	2 56	0 010	2 56	0 001	0
	As above in 0 02 N CaCl₂	3 85	0 010	3 85	0 001	0
Parchment paper	Between two membranes 2 per cent lecithin solution in 0 02 N KCl	1 31	<0 001	1 31	<0 001	0
	As above in CaCl₂ of 0 005–0 068 N	No difference in conductivity at low and high frequencies detectable Capacity at both frequencies <0 001 until N = 0 028 At highest concentration C 560 = 0 02, C 5000 = <0 001				

detected in these protein-collodion membranes even when treated with $CaCl_2$, HCl, or NaOH or electrodialyzed

In the second series of investigations gelatin was used for preparing membranes or as a medium for blood proteins, genuine or heat-denatured   It has been mentioned previously that 20 per cent gelatin membranes treated according to Collander (15) with 10 per cent formaldehyde and containing up to 2 per cent lipoids were not polarizable   These results could not be modified by using a thicker gelatin membrane, a membrane containing less water, or one hardened ultimately in 36 per cent formaldehyde   Formaldehyde was added to the fluid used in the conductivity measurements   Furthermore, a membrane consisting of a homogeneous layer formed by a concentrated solution of sodium caseinate on filter paper, did not show any polarizability even when treated with HCl or formaldehyde (Table VIA)

On account of these numerous failures, a fundamental change in the method of preparing the membranes was advisable

It is well known that certain colloids are adsorbed on surfaces, this process being sometimes followed by reversible or irreversible denaturation as in the case of proteins (16)   Experiments were instituted in which protein (egg albumin) was adsorbed on the sintered glass plate of a Jena glass crucible   Sintered glass crucibles No 3 and No 4 were used in which the sizes of the pores were, according to the manufacturers, respectively 20–30 μ and 5–10 μ

In preliminary experiments it was shown that the insertion of a sintered glass plate into the pathway of an alternating current did not lead to polarization after the whole system had been filled with 0 1 N KCl

With suction a 1 per cent solution of egg albumin was filtered through the glass plate   The filtration rate decreased very soon   In one experiment the interval between two drops was 720/5 seconds after 30 minutes, 1310/5 seconds after 1 hour and 20 minutes, practically nothing for the protein solution as well as for distilled water after 2 more hours

In order to measure the polarizability of such a prepared filter plate, the following apparatus was devised   A wire gauze platinum electrode (diameter = 20 mm) was introduced into a funnel in such a way that the electrode plate was tightly fixed in a position parallel to the glass

plate of the crucible   The funnel was then filled to the brim with 0 1 N KCl   The crucible was inserted tightly into the funnel with the help of a rubber band   In order to exclude air bubbles, the bottom of the crucible had been filled previously with a 10 per cent gelatin solution containing 0 1 N KCl   The crucible itself was filled with 0 1 N KCl, and an electrode similar to the one described was immersed in the crucible   A clamp held the electrode fixed so that its plate remained parallel to the first electrode and to the glass plate   The distance between the plates was 30 mm   The conductivity of such a system was about 1 45 10⁻² reciprocal ohm   No trace of polarization could be detected with the usual conductivity measurements

These experiments were repeated with different proteins, serum albumin, and casein, and with samples of these proteins acidified to increase the adsorption or treated with absolute alcohol or heat coagulated   Still no polarization of the filter plate was obtained

Furthermore, it was not possible to render the sintered glass plate polarizable by treatment with colloid aqueous lecithin solutions or with alcoholic lecithin solutions, even if it was allowed to remain in the crucible for 12 hours   Even under the coagulating influence of HCl, $CaCl_2$ or $La(NO_3)_3$, it was impossible to cause a noteworthy choking of the glass plate (Table VI B)

Finally experiments were begun in which biological conditions were imitated by using membranes containing both proteins and lipoids Sintered glass plates prepared with egg albumin in the manner already described were treated with alcoholic egg lecithin solutions   The crucibles were filled with 0 1 N KCl and allowed to stand for 12 hours After this time, measurements of the conductivity disclosed a decided increase in resistance and capacitance and a definite polarization of the plate, corresponding to a Δ of 5 8 per cent

The difference in pore size of the original sintered glass plate seemed to be of no importance

Confirmation of these results was sought by a different method According to Loeb (17), every impediment to the movement of an ion calls forth a potential difference   To test in this respect the action of the original glass plate and one containing adsorbed protein and lipoid, measurements of potential across those plates were made Since direct current was to be used, non polarizable silver silver

chloride electrodes prepared according to Langelaan (18) replaced the platinum electrodes, and the crucible containing one of the electrodes was fixed with a clamp and dipped into a glass vessel which contained the other electrode   The crucible and the glass vessel were filled with 0 1 N KCl   The air bubble under the crucible was removed by aspiration   A compensation box and a string galvanometer as zero-point instrument were used in measuring the potential necessary to

TABLE VI B

Various Agents Which Did not Render the Sintered Glass Plate Polarizable

Adsorbed material	Conductivity at 560 cycles	C	Conductivity at 5000 cycles	C	Δ
I egg albumin	4 82 10⁻³	0 005	4 82 10⁻³	0 001	0
II " "	1 53 10⁻³		1 53 10⁻³		0
I after heating to 100°	4 42 10⁻³	0 005	4 42 10⁻³	0 001	0
I + HCl	—		—		0
II treated with alcohol	7 23 10⁻³	<0 001	7 23 10⁻³	<0 001	0
Casein in NaOH neutralized on filter plate	1 27 10⁻³	<0 001	1 27 10⁻³	<0 001	0
As above, treated with formaldehyde	5 43 10⁻⁴	<0 001	5 43 10⁻⁴	<0 001	0
Casein in HCl	—		—		0
Casein in HCl treated with alcohol	2 28 10⁻³	<0 001	2 28 10⁻³	<0 001	0
Serum albumin	1 45 10⁻³	<0 001	1 45 10⁻³	<0 001	0
Lecithin in aqueous solution					
Same as above + 0 1 N CaCl₂					
Lecithin solution + 0 02 N La(NO₃)₃	No clogging of glass filter				
Lecithin solution + 0 006 N HCl					
Alcohol c lecithin solution	6 03 10⁻⁴	0 002	6 03 10⁻⁴	<0 001	0

compensate that of the system   The results of a series of investigations are summarized in Table VII, indicating that the potential called forth by the protein-lipoid-glass membrane amounts to 4 mv

These results confirm the results obtained by the measurements of the Δ and point to the usefulness of the latter method

With crucibles prepared in the aforesaid way, some experiments of basic interest for biological problems were made

The KCl was replaced by other solutions of the same normality

The Δ was measured after equilibrium had been reached   The results are summarized in Table VIII   According to these figures, the polarization is much increased if potassium is replaced by magnesium   The narcotic effects of magnesium salts are as well known as their precipitating effect upon lecithin sol which is used for analytical

TABLE VII

Polarization of a Protein Lipoid-Glass Filter Plate

	Ag AgCl-electrodes		Platinum electrodes		
	Galvanometer readings	mv	Conductivity at 560 cycles	Conductivity at 5000 cycles	Δ
					per cent
Electrodes alone ($= A$)	$\frac{25-15}{5}$	2	—	—	—
A + glass filter plate ($= B$)	$\frac{30-15}{5}$	3	$4\ 42\ 10^{-3}$ $C = 0\ 001$	$4\ 42\ 10^{-3}$ $C = 0\ 001$	0
B + adsorbed protein lipoid	$\frac{50-15}{5}$	7	$2\ 74\ 10^{-3}$ $C = 0\ 028$	$2\ 89\ 10^{-3}$ $C = 0\ 005$	5 8

TABLE VIII

Action of Various Ions on the Polarizability of Protein Lipoid-Glass Filter Plate

0 1 N concentration of	At 560 cycles		At 5000 cycles		Δ
	Conductivity	C	Conductivity	C	
					per cent
KCl	$2\ 52\ 10^{-3}$	0 028	$2\ 65\ 10^{-3}$	0 005	5 13
KBr	$2\ 60\ 10^{-3}$	0 030	$2\ 77\ 10^{-3}$	0 010	6 50
MgCl$_2$	$1\ 72\ 10^{-3}$	0 038	$1\ 88\ 10^{-3}$	0 005	9 25
AlCl$_3$	$6\ 03\ 10^{-4}$	0 010	$6\ 46\ 10^{-4}$	0 001	7 05
La(NO$_3$)$_3$	$6\ 81\ 10^{-4}$	0 017	$7\ 42\ 10^{-4}$	0 003	9 0
HCl	$9\ 94\ 10^{-4}$	0 011	$1\ 05\ 10^{-3}$	0 003	5 8
NaOH	$1\ 21\ 10^{-3}$	0 003	$1\ 23\ 10^{-3}$	0 001	1 6

purposes   Narcotics generally call forth a decrease in permeability of cell surfaces (19)   From valency alone the effect of an ion on the polarization of these plates cannot be predicted, lanthanum proving more effective than aluminum   A similar observation has already been made in *in vitro* experiments on brain tissue   Hydrochloric acid

affected the polarizability only slightly more than potassium chloride, while sodium hydroxide decidedly caused a drop in polarization Analogous observations have been made on animal brain tissue *in vitro* (1) Furthermore, the specific effects of bromides were studied The effectiveness of bromides in restoring the polarizability of swollen brains in *in vitro* experiments has been ascertained before (1) In another paper (20) a direct action of the bromide on the lecithin molecule was demonstrated In a series of experiments it was shown that the polarizability of a protein-lipoid-glass plate in equilibrium with potassium chloride was further increased when potassium chloride was replaced by an equinormal solution of potassium bromide

TABLE IX

Influence of Various Adsorbed Material on the Polarizability of Glass Filter Plate

Material	Conductivity at 560 cycles	C	Conductivity at 5000 cycles	C	Δ
					per cent
Starch	1 29 10⁻²	0 030	1 29 10⁻²	<0 001	0
Starch + protein	1 28 10⁻²	0 022	1 28 10⁻²	0 002	0
Starch + lecithin	6 04 10⁻²	0 030	6 29 10⁻²	0 008	4 2
Protein + alcohol soap solution	1 14 10⁻²	0 020	1 14 10⁻²	<0 001	0

Finally the influence of a lipoid-soluble agent on polarizability was determined If ethyl alcohol was replaced by amyl alcohol as a solvent for lecithin, protein-glass filter plates prepared with the amylic solution showed a higher Δ than the ones prepared with the ethylic solution The narcotic effect of amyl alcohol is twelve times greater than that of ethyl alcohol In recent experiments (21) it was shown that the salt-binding capacity of lecithin was reduced by ethyl alcohol and practically abolished by amyl alcohol

In most of these experiments the reaction of the composite membrane appears to be determined by the reactions of the lipoid component The rôle of proteins seems to be of a more mechanical nature, *e g* that of decreasing the size of the pores in the sintered glass plates This view is supported by experiments showing that proteins can be replaced by soluble starch The molecular weight of starch is 100,000

according to Samec (22)   Starch alone does not cause polarization of the sintered glass plate

It has not been possible so far to accomplish these results, if the lipoids were replaced by soluble starch or salts of fatty acids (Table IX) This failure is interesting in view of the many theories stressing the importance of lipoids in biological membranes

SUMMARY

1 Collodion membranes of high polarizability and low resistance can be obtained either by addition of certain ether soluble substances such as phosphatides, olive oil, mastix, and gum benzoin, to the collodion or by drying collodion membranes for a limited time under pressure

2 The permeability of membranes of different polarization has been measured by means of conductivity methods

3 Sintered glass filter plates of Jena glass crucibles on which proteins and lipoids have been adsorbed show polarization   It could be shown that some narcotics which react with lecithin cause an increase in polarization of the protein lipoid glass system   Substitutions of the protein but not of the lipoid were possible, without causing a decrease in the polarizability of the membranes

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THE QUANTUM YIELD OF HYDROGEN AND CARBON DIOXIDE ASSIMILATION IN PURPLE BACTERIA

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INTRODUCTION

Photochemical carbon dioxide reduction in the purple bacteria differs from the process of photosynthesis in green plants in that a reducing substance other than water is required (van Niel and Muller (1931)) Sulfur compounds (van Niel (1931)), organic acids (Gaffron (1933, 1934, 1935 b), Muller (1933)), and gaseous hydrogen (Roelofsen (1934, 1935), Gaffron (1935 a) and van Niel (1935)) may be used The present discussion concerns the reaction which takes place in suspensions of *Streptococcus varians* when irradiated in the presence of H_2 and CO_2

For the quantitative study of a photochemical reaction the amount of chemical change produced by a known quantity of light absorbed by the reacting substance or sensitizing pigment must be measured The investigation of quantum yields of processes in live cells has three aspects the optical, the chemical, and the physiological The optical side of the problem is concerned with the determination of the amount of light absorbed per unit time under the experimental conditions by the reacting substance or by the sensitizing pigment, and with making certain that scattering and absorption by other colored substances do not obscure the result In the classical experiments of Warburg and Negelein (1922, 1923) on photosynthesis in the alga *Chlorella vulgaris*, thick suspensions of cells were used so that absorption was complete For the present work it was found impossible to use this arrangement so thin suspensions of measured transmissions were employed (Wurmser (1926))

The amount of chemical change was measured with a slightly modified differential manometer

On the physiological side it is essential to insure that the cells are

all actively metabolizing and that the experimental conditions are so chosen that the maximum possible chemical change is produced for a given amount of light

As there is little free energy change in going from the gases $2H_2 + CO_2$ to the probable product (presumably similar to a carbohydrate), it is of especial interest to compare the quantum yields of this reaction with photosynthesis in algae where the bound energy is large

EXPERIMENTAL

Physiology of the Hydrogen and Carbon Dioxide Assimilation

Before quantum efficiency can be measured and considered to have a real meaning it must be established that the rate of reaction under the conditions of the experiment is limited by the light intensity and by that alone   Preliminary experiments were made to determine the most favorable methods of growing and handling the bacteria so that essentially all the bacteria were alive and active at the time of the experiment   The factors of H_2 pressure, CO_2 pressure, pH, temperature, light intensity, and time interval of the experiment were studied and the effects will be summarized here

A pure culture of *Streptococcus varians* designated as *C*11 was obtained through the courtesy of Professor C B   van Niel   Stock cultures were maintained as stabs in agar with peptone or in the yeast extract medium here described   The cultures for daily use were grown overnight at 35°C over an Osram show-case lamp immersed in the thermostat   Flasks of the type used by Warburg for *Chlorella* cultures were filled with 100 cc of sterile medium and inoculated with 2 cc of a similarly grown inoculating stock culture kept under pure culture conditions at 0°C   The air was blown out of the flask with a stream of 5 per cent CO_2 in argon and it was sealed by attaching the same rubber tube to both openings which were plugged with cotton   Such anaerobic cultures were brown while aerobic ones were red   The medium had the following composition

KHCO$_3$	0 5	per cent (0 05M)
NH$_4$Cl	0 1	" "
KH$_2$PO$_4$	0 05	" "
MgCl$_2$	0 02	" "
Yeast autolysate[1]	2 0	" "

In the morning the suspension was centrifuged half an hour at 6,000 R P M (radius about 25 cm) and the culture solution decanted off   The bacteria were

---

[1] Yeast autolyzed 2 days at 50°C with an equal volume of water, then boiled, centrifuged, decanted, and sterilized

then taken up in 5 cc of tap water and centrifuged at 10,000 R P M (radius about 10 cm) for 1 minute, which threw down a sediment produced by the culture medium, leaving the bacteria suspended 10 minutes sufficed to pack the bacteria down, then they were resuspended in the desired medium for the experiment, generally 0 05 M KHCO₃ in tap water Control experiments demonstrated that the rate of assimilation by centrifuged and non-centrifuged cells was identical and

TABLE I

The Homogeneity of the Bacterial Culture

Fractions separated after 12 minutes of centrifuging at 3000 R P M.

Fraction	Relative rate of H₂ assimilation per c.mm cells
1	0 82
2	0 98
3	0 84
4	0 99

TABLE II

Utilization of H₂ in the Light
Conical Vessels over Show-Case Lamp at 25°C

Main space   3 5 cc. 0 033M KHCO₃ containing 240 c mm bacteria in each
Side arm   0 1 cc. 0 1M K butyrate in each

Gas space		5 per cent CO₂/H₂	5 per cent CO₂/A
min		*mm*	*mm*
5	Light	−22	0
5	'	−22	0
5	Dark	+2	+3
5		+2	+2
	Butyrate added from side arm		
5	Dark	+1	+2
5	Light	−29	−4
5		−27	−3

that bacteria of differing activity could not be fractionated by partial packing, as is illustrated by the data in Table I   Cell volumes of suspensions were measured in hematocrit tubes after 10 minutes of rotation at 10 000 R P M

That assimilation of CO₂ and H₂ takes place in the light but not in the dark is apparent in Table II   The assimilation of CO₂ in the presence of butyrate is also shown   Throughout this work it was

assumed that two molecules of hydrogen were used for one of CO_2, as was shown to be the case with related bacteria by Gaffron (1935 a)
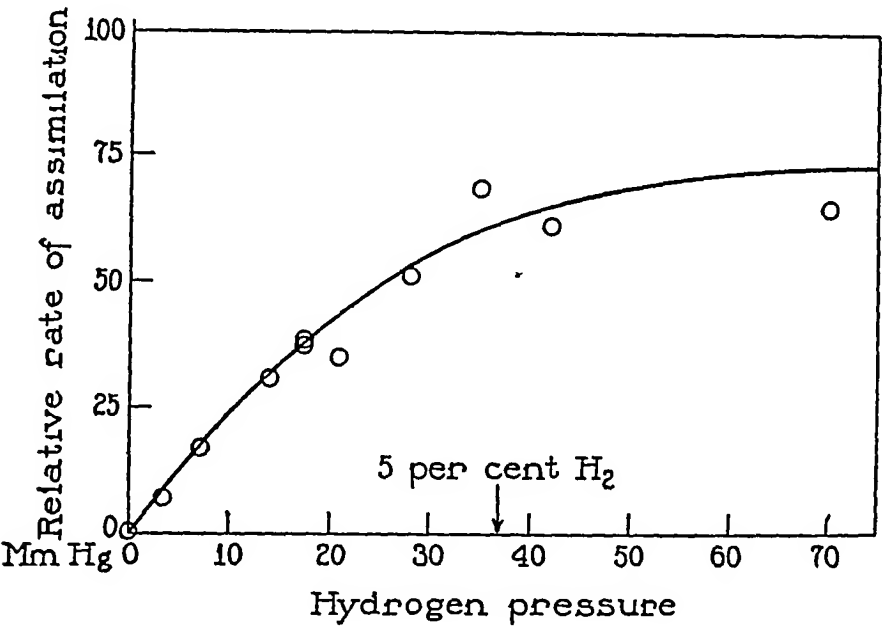
FIG 1 Effect of H_2 tension on rate of H_2 assimilation   Conical vessels with 3 cc yeast extract mineral growth medium 0 02 M $KHCO_3$ 114 c mm cells Osram show-case lamp below vessels   Temperature 25°C   Various amounts of H_2 in argon   5 per cent CO_2 present in all   Rate in 95 per cent H_2 taken as 100

TABLE III

Effect of CO_2 Tension on Rate of Assimilation in Phosphate Buffers

Various amounts of CO_2 in H_2 in gas phase   Conical vessels over show-case lamp at 25°C

CO_2	pH	Rate
per cent		
(0)	7 35	3 9
1	7 55	27 1
5	7 35	34 8
10	7 25	32 3

On this basis pressure readings were translated to cubic millimeters of hydrogen

In Fig 1 we see the relation between rate of assimilation and H_2

pressure   Half the maximal rate is reached at a tension of 27 mm
H_2, equivalent to roughly 4 per cent of H_2 in the gas phase   The
quantum yield experiments were done with 95 per cent H_2, that is a
pressure of 700 mm Hg corrected for water vapor   From Table III,
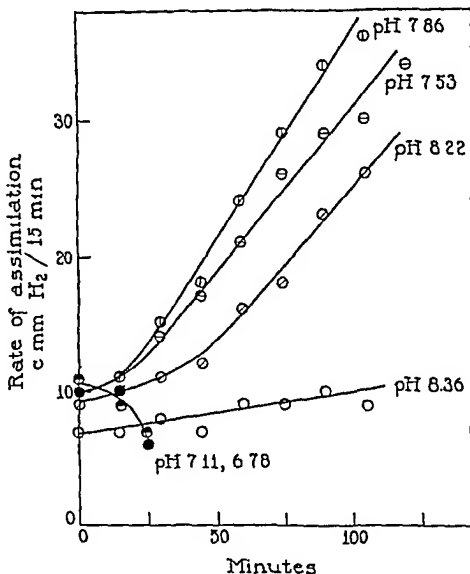
FIG 2   Rate of H_2 assimilation at different pH values as a function of time
Conical vessels temperature 25°C   show-case lamp 5 per cent CO_2 in H_2, pH
controlled by various dilutions of $KHCO_3$ and measured with a glass electrode

it is seen that 5 per cent CO_2 is more than adequate for saturation
with CO_2

Fig 2 shows the rate of assimilation at different pH values with 5
per cent CO_2/H_2 as a function of time   The marked increase of rate
with time in the favorable range pH 7 5 to 7 9 led to the two experi-
ments shown in Figs 3 and 4   The former indicates that a dense
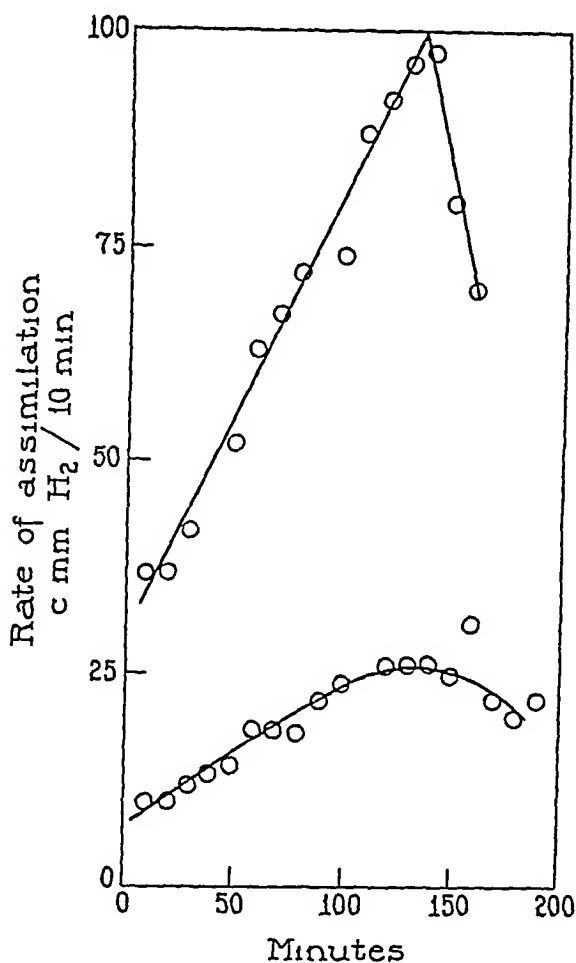
FIG 3 Rate of H_2 assimilation as a function of time in thick and thin suspensions

	Cell volumes	Initial rate	Slope
Top curve	216	27	5 5
Bottom curve	72	7	1 5
Ratio, top/bottom	3 0	3 9	3 7

Both these suspensions show a decrease in rate at the same time whereas the amount of assimilation and amount of light absorbed is widely different in the two vessels   This suggests that the changes may be due to something like an osmotic or salt effect rather than CO_2 exhaustion or accumulation of photosynthetic products   There was practically no change in cell volume during the experiment

culture increases in rate just as rapidly as a thin one   The effect is not due to growth of bacteria as the cell volume does not increase during the experiment   This increase of rate is dependent on the salt content (Fig 4) as well as the pH   Still more striking time effects influenced by salts have been found by van Niel (1936) with *Thiorho daceae*   The nature of this phenomenon is not yet clear, although its implications are serious when absolute measurements are needed

The interesting behavior of the assimilation process with respect to light intensity is shown in Figs 5, 6, and 11 where it is seen that the
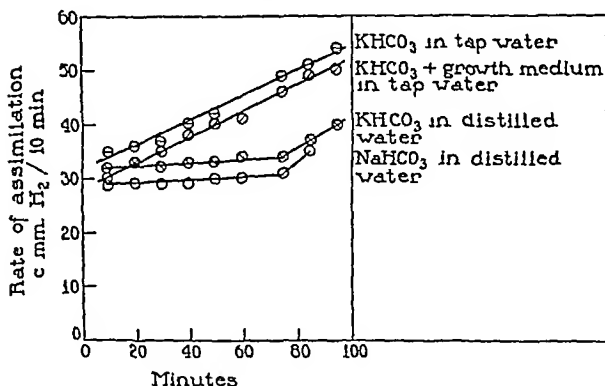
FIG 4 Rate of H_2 assimilation in various media   After the experiment the two upper curves had more motile bacteria than the lower ones

curve is sigmoid and more markedly so at high temperatures   Over thirty experiments confirmed this relationship

It follows from these curves that dense suspensions absorbing all the light cannot be used for efficiency experiments because in a totally absorbing suspension different cells receive greatly different intensities   However, for the curves of Fig 6 a dense suspension was used and total absorption assured by silvering the outside of the vessel It is seen that the near infrared is much more effective per calorie absorbed   This is consistent with more detailed work on the spectral

sensitivity of a photochemical reaction in *Spirillum rubrum* not yet published   It was therefore evident that light in this region and a partial absorption method would have to be used
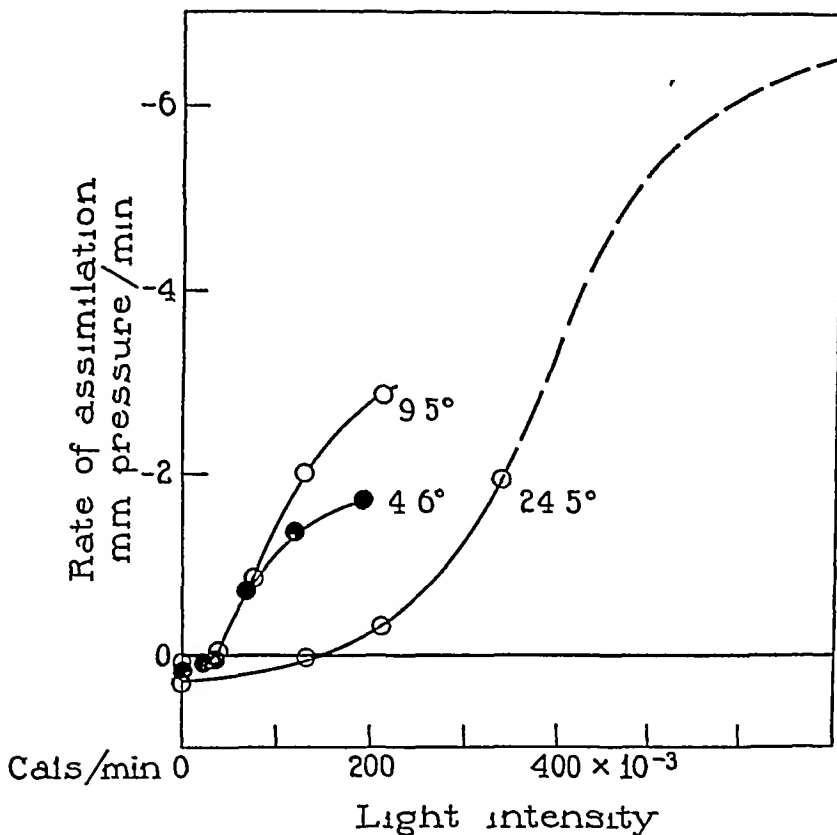
FIG 5   Rate of H₂ assimilation at different temperatures as a function of light intensity   Large silvered differential manometer, total absorption   Open circles, 250 c mm cells in 25 cc of growth medium   Filled circles, 500 c mm cells, 700–900 mμ isolated from filament lamp with Schott filters, BG3 + RG2 + 6 cm water   Factor of 0 5 taken for the transmission of the 15 cm of thermostat water   Intensity scale indicates total incident light in cals /min over the vessel area of 21 2 cm²   At 9 5° K_H = 2 84   Apparent γ = 11 5 quanta/H₂

Measurement of Light Absorption by Bacterial Suspensions

It was not possible in the spectral region used to estimate the absorption of a suspension of cells by measuring the absorption of the extracted pigment because the main band in the infrared is shifted very greatly in alcohol   Therefore, we have used simply the absorp-

tion of the dilute suspension as measured directly with a photocell behind a thin absorption vessel Various experiments described here appear to justify the conclusion that one obtains, under the specified
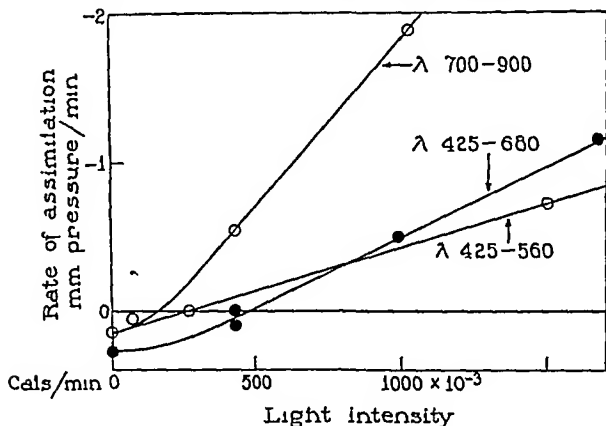
Fig 6 Rate of H_2 assimilation with different spectral regions as a function of light intensity Large silvered differential manometer, total absorption, 25 cc. bacterial suspension as grown 42 amp Osram Kino lamp Intensity scale as in Fig 5

Approximate wave length region	Filters
700–900	Schott BG3 + RG2 + 6 cm water
425–680	3 cm 25 per cent FeSO₄ in 10 per cent H_2SO_4 + 6 cm. water
425–560	1 cm 2 5 per cent CuSO₄ − NH₃ + 6 cm 25 per cent CuSO₄

conditions, an approximation to the light taken up by the active pigment

In a rectangular vessel filled with a dilute suspension of colored cells, let

I_0 = total entering light (corrected for glass reflection),

I = total light emerging from the opposite face

I = total light emerging from the four sides,

I_r = light reflected from the face of incidence,

I_a = total light absorbed,

I_p = light absorbed by the photochemically active pigment

Evidently,

$$I_0 = I + I_s + I_r + I_a$$

When the conditions are such that scattering and reflection from the suspension, I_s and I_r, are vanishingly small the absorbed light is simply

$$I_a = I_0 - I$$

and if only the photochemically active pigment absorbs in the wave length region considered

$$I_p = I_0 - I$$

This simple state of affairs is believed to have been realized in the efficiency experiments done with infrared light

Control experiments with visible light were made in thick vessels to see if the scattering of light was significant in relation to pigment absorption   The bacteria have a sharp absorption band at 590 mμ due to the green pigment

For the purpose of measuring the light coming out the different faces of rectangular vessels, sodium light, 589 mμ, was measured with a flat surface photocell placed in contact with the glass wall of the vessel   As visible light was used the reflection due to the cells could be approximately determined by comparison with a magnesium oxide surface illuminated by a beam which was dimmed by filters till it appeared equal in brightness to the suspension viewed at a small angle from the direction of the light   The transmission of the filter combination was then determined with the photocell

Table IV shows the approximate percentage of visible light emerging from the various faces of the vessel and hence the absorption within the suspension   In the large vessel, an appreciable absorption by the bleached cells is noticed but this is smaller in the thinner vessel   The greater apparent scattered light in the bleached suspension is attributed to its lack of absorbing pigment

The amount of scattered light lost at the sides is about 10 per cent under these conditions   In thinner vessels—0 1, 0 2, and 0 5 cm —as used for the measurements described below it would be only a few per cent and with infrared vanishingly small

Evidence of far less scattering in infrared (852 mμ + 894 mμ) than in sodium light was obtained by varying the distance from vessel to photocell   With visible light the photocell current fell rapidly as the distance was increased, so all readings had to be made with the glass vessel in contact with the photocell   With infrared the cell could be moved a centimeter away before a fall in readings was obtained

Small but regular deviations from Lambert's and Beer s laws are shown in Table V for visible light passing through cell suspensions Light from a monochromator, wave length band 580–600 mμ, was used

Fig 7 shows the apparatus with which absorption measurements were made in the infrared

TABLE IV

The Distribution of Light Leaving a Bacterial Suspension Illuminated from One Side
(*589 mμ*)

| | Vessel 2 0 × 2 0 × 2 0 cm | | | |
| c mm per cc | $c = 10$ | | $c = 5$ | |
	Normal	Bleached	Normal	Bleached
Incident I_0	100	100	100	100
Transmitted I	15	36	37	54
Reflected I	2	5	2	4
Scattered I	16	38	11	20
Absorbed* I	67	21	50	22

| | Vessel 1 5 × 1 58 × 0 8 cm † | | | |
| c mm per cc | $c = 10$ | | $c = 5$ | |
	Normal	Bleached	Normal	Bleached
Incident I_0	100	100	100	100
Transmitted I_1	51	67	72	83
Reflected I	2	4	2	4
Scattered I	10	19	7	9
Absorbed* I	37	10	19	4

* Absorbed was taken as $I = 100 - I - I - I$

† Light enters 1 5 × 1 58 face

I_0 has been reduced 4 per cent to allow for air glass reflection of entering light No reflection correction was applied for partial reflection of the light leaving the suspension since it is mainly absorbed

In Tables VI and VII are sample measurements of transmissions of bacterial suspensions in the concentrations used for the manometric experiments Beer's and Lambert s laws are followed within the rather large error   Transmissions of the suspensions used in the manometer were always measured in three vessels of 0 097, 0 202 and 0 50 cm and plotted against the thickness As the average thickness of the liquid in the manometer vessel was 0.293 cm. the transmission at this value was determined by interpolation and the fraction absorbed, F, = $(1 - T_{0\ 293})$ With the density and thickness of suspensions used, the absorption of a suspension of bleached bacteria was identical with that of water for infrared

"Sperrschicht" photocells were used, one with a maximum in the visible for the work with 589 mμ, and another of far less sensitivity with a maximum at about 850 mμ for the work in that region, from Dr B Lange, Berlin-Dahlem
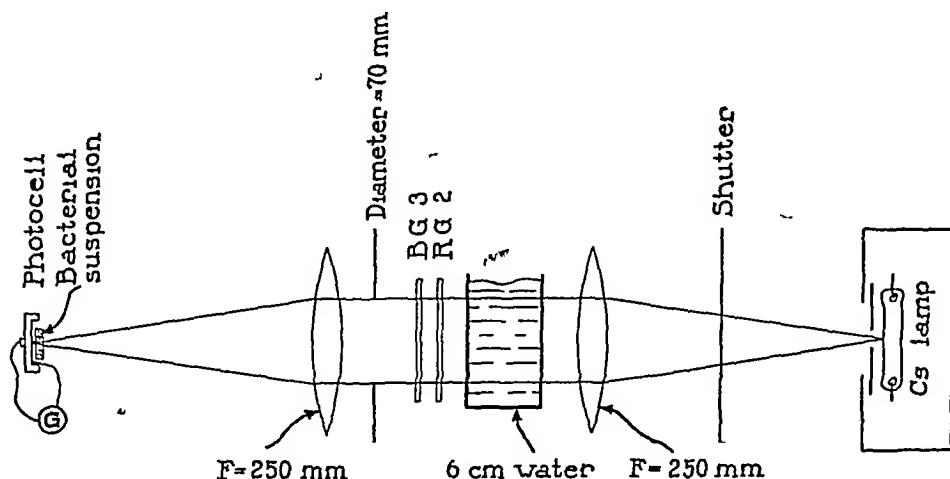
FIG 7 Apparatus for absorption measurements with 852 + 894 mμ

TABLE V

Approximate Applicability of Beer's and Lambert's Laws to Cell Suspensions

β* value for various thicknesses and cell concentrations for 580–600 mμ

Concentration	β			
	1 93 cm	1 07 cm	0 49 cm	0 202 cm
c mm /cc				
20 2	—	0 0247	0 0232	0 0213
10 1	0 0253	0 0232	0 0222	0 0205
5 05	0 0242	0 0224	0 0222	
2 52	0 0232	0 0236		

*$\beta = \dfrac{\log_{10} I_0/I}{cd}$ The spectral region was isolated by a monochromator

The Light Source and Optical System

An Osram[2] caesium tube was used for a line source   When operated at 3 amperes in series with a choke coil and a resistance on the 220 volt A C circuit it

[2] Through the kindness of Dr Teissing of the Physikalisch-Technische Reichanstalt an Osram caesium tube was placed at our disposal

Various sources with appropriate filters for isolation of single lines in the near

gave the spectrum shown in part D of Fig 8 Nearly all the intensity is
in the lines at 852 mμ and at 894 mμ Schott filters BG3 and RG2 with 6 cm of
water were used to remove the visible and far infrared If desired Agfa filter
No 85 may be used to remove completely the weak lines of shorter wave length
than 840 mμ Didymium glass also has useful bands in this region Fig 9
shows the spectrogram of the bacterial suspension From a comparison of Figs 8
and 9, it appears that 852 mμ and 894μ are absorbed to roughly the same extent

TABLE VI

Sample Measurements of Absorption of a Cell Suspension with 852 + 892 mμ

Thickness	I_0 Bleached	I Normal	βc
cm			
0 50	9 1	4 8	0 56
0 202	9 1	6 7	0 65
0 097	9 2	8 0	0 63
0 56	9 3	4 4	0 58
0 202	9 4	7 3	0 55
Average			0 595

TABLE VII

Sample Measurements of Absorption of Cell Suspensions with 852 + 894 mμ

Thickness	Concentration	I_0 Bleached	I Normal	T	T calc $\beta = 0$ 080	β
cm	mm /cc					
0 50	10	8 5	3 6	0 42	0 40	0 074
0 202	10	8 5	5 4	0 64	0 69	0 097
0 097	10	8 5	7 2	0 85	0 83	0 074
0 50	5	10 5	6 6	0 63	0 63	0 081
0 202	5	10 5	8 4	0 80	0 83	0 096
0 097	5	10 4	9 7	0 93	0 92	0 062
Average						0 080

by the bacteria and both lines fall within the same absorption band It is felt
that this tube is a satisfactory source for work in this region which falls between
the much studied visible spectrum and the now popular longer wave infrared

When used with the optical system shown in Fig 10 the available energy was
200 × 10^{-5} cals /min when the tube was new but decreased to one third of this

infrared visible and ultraviolet spectrum are described in pamphlets issued by
the Osram Studiengesellschaft Berlin
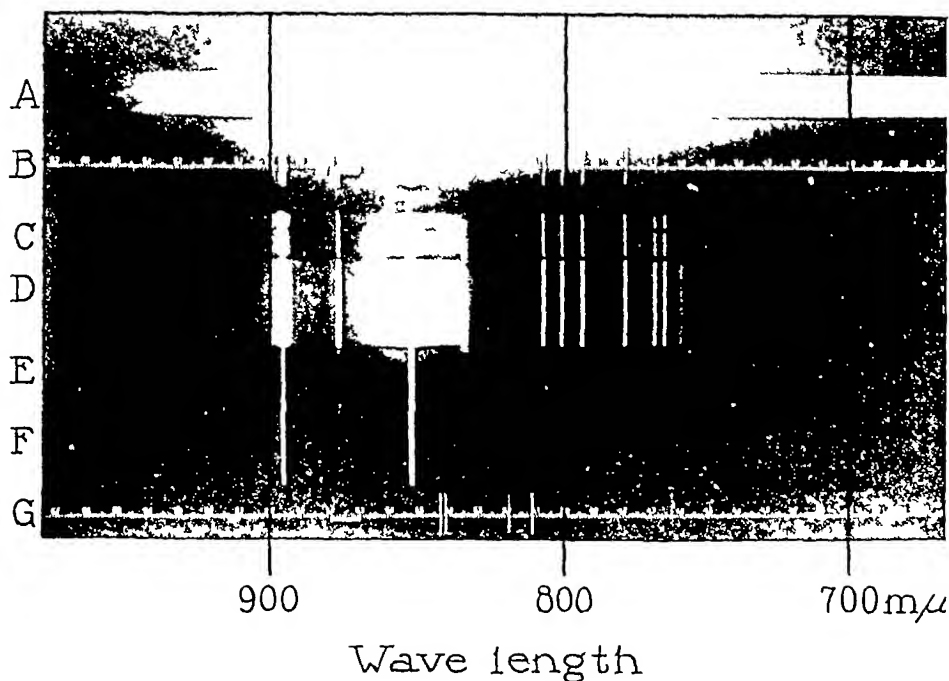
FIG 8 Near infrared spectrogram of the caesium lamp   Taken with Zeiss spectrograph for chemists converted for infrared work with the grating   The wave length region has been shifted 300 mμ in relation to the scale on the plate The number at the bottom shows correct wave length   Plate, Agfa "850" Schott filter RG2 used to cut off second order spectrum

	Dia phragm	Slit width	Lamp amper age	Ex posure
	cm	mm		min
A Filament lamp	—	0 2	10	1
B Caesium "	1 5	0 1	3 0	5
C " "	1 5	0 1	3 0	20
D " "	—	0 1	3 0	15
E " "	—	0 1	2 0	15
F " "	—	0 1	1 0	75
G Na tube, just started, showing A lines	1 5	0 1	2 0	10

after running between 50 and 100 hours   It was allowed to warm up at least half an hour before use to prevent intensity change during the measurements   Large energies were necessary because the sigmoid character of the intensity curve made
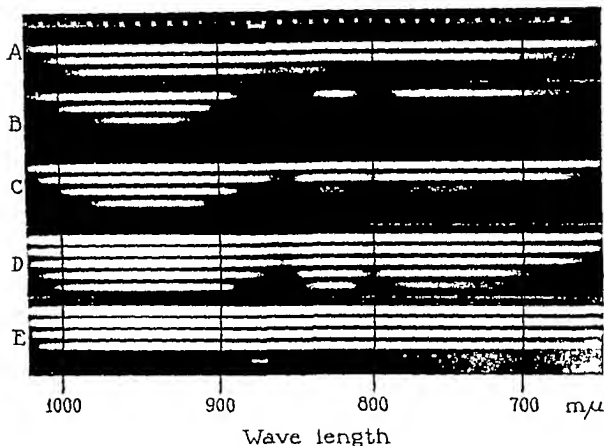
Fig 9 Infrared absorption bands of *Streptococcus tarians* Plate 950 Agfa
A Filament lamp Slit = 0 15 mm Exposures 4 2 1 min for comparison
   with spectra B and C
B Same with 0 2 cm bacterial suspension in tap water before slit 70 c mm
   cells per cc Exposures 16 8, 4 2, 1 min
C Same with 0 1 cm of same suspension Exposure 16 8 4 2 1 min
D 0 5 cm bacterial suspension in yeast extract as grown Exposures 16, 8
   4 2, 1 min
E Filament lamp as used for spectra D Exposures 4 2 1 0 5 min

it essential to carry out measurements over a large range in order to find the
maximum slope

Quantum Yield Experiments

7 cc of the suspension of bacteria in 0 05 M KHCO₃ in tap water was
placed in one vessel of a differential manometer and some of the same
suspension set aside for the transmission determinations The control
vessel contained 7 cc of the bicarbonate solution without bacteria
On the assumption that the pressure change was due to 2H + CO

disappearing, the cathetometer readings were reduced to cubic milli-
meters of H_2 by the following formulae

$$h = \text{pressure change in mm Brodie solution}$$
$$X_{H_2} = c \text{ mm } H_2$$
$$k_{H_2} = \text{vessel constant for } H_2$$
$$k_{CO_2} = \text{vessel constant for } CO_2$$
$$X_{H_2} = h \times K_{H_2}$$
$$K_{H_2} = \frac{k_{CO_2} \times k_{H_2}}{0\,5k_{H_2} + k_{CO_2}} \approx 1\,72$$
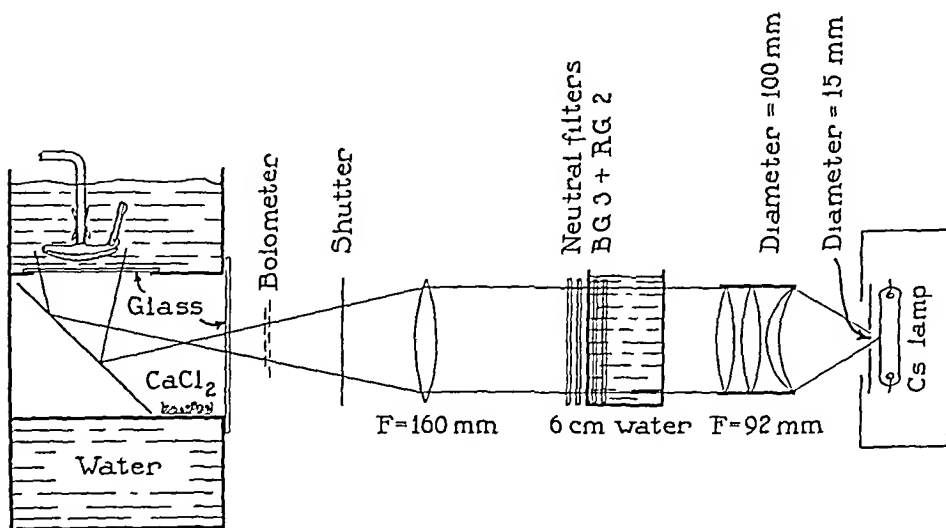
FIG 10 Apparatus for photochemical measurements

The vessel constants, k_{H_2} and k_{CO_2}, were obtained by the formula
given by Warburg (1926)

The vessels (Fig 10) were thin and rounded on the bottom to keep
the liquid from climbing up on the sides too much during shaking
The dimensions were

Area of bottom of experimental vessel = 23 8 cm ^{2}
Volume of experimental vessel = 19 37 cc = 19,370 c mm
Volume of control vessel = 23 15 cc = 23,150 c mm
Area of capillary cross-section = 0 103 mm ^{2}

All the experiments described in this section were done at 9 2°C , as

at higher temperatures the initial flat portion of the intensity rate curve was exaggerated and at lower temperatures the rates were too small to measure accurately   Readings were made for two 5 minute periods at each light intensity after 5 minutes adaptation

In Fig 10 is shown the arrangement for illuminating the vessel The single lens produces a round, sharp, evenly illuminated image of the condenser lens at the vessel plane, the area B of which is computed from the measured diameter   The thermostat was designed to give as short a light path as possible through the water which absorbs appreciably at 850 mμ The dish of $CaCl_2$ keeps the air in the chamber dry, eliminating fogging of the glass surfaces   Absolute energy measurements were made either at the beginning or end of the experiment with a bolometer which measured all the light in the beam focussed upon it with a shorter focus lens than that used to illuminate the vessel   The intensity at the plane of the vessel is given by

$$I_1 = \frac{RT_w \times 21\,300}{\Omega B} \times 10^{-2} = \text{cals/min/cm}^2$$

where

R = reflection correction for glass between bolometer and vessel
T_w = transmission of 1 cm water for 852 mμ = 0 96
B = area of light circle at vessel
21 300 $\times$ 10^{-3} = bolometer calibration factor determined with a Hefner lamp
Ω = resistance in ohms in circuit to bring the galvanometer back to zero during illumination of bolometer

The intensity could be diminished to nearly any desired value with a set of neutral glass filters calibrated bolometrically with this light

Measured rates of assimilation in millimeters of Brodie solution per 5 minutes were plotted against light intensity (Fig 11)

The Einstein, (mole quantum), is

$$1 \text{ Einstein} = Nh\nu = \frac{Nhc}{\lambda} = \frac{6\,06 \times 10^{23} \times 6\,55 \times 10^{-27} \times 3 \times 10^{10}}{852 \times 10^{-7}}$$

$$= 1\,40 \times 10^{12} \text{ ergs}$$
$$= 33\,500 \text{ cals}$$

$$(1 \text{ cal} = 4\,18 \times 10^7 \text{ ergs})$$
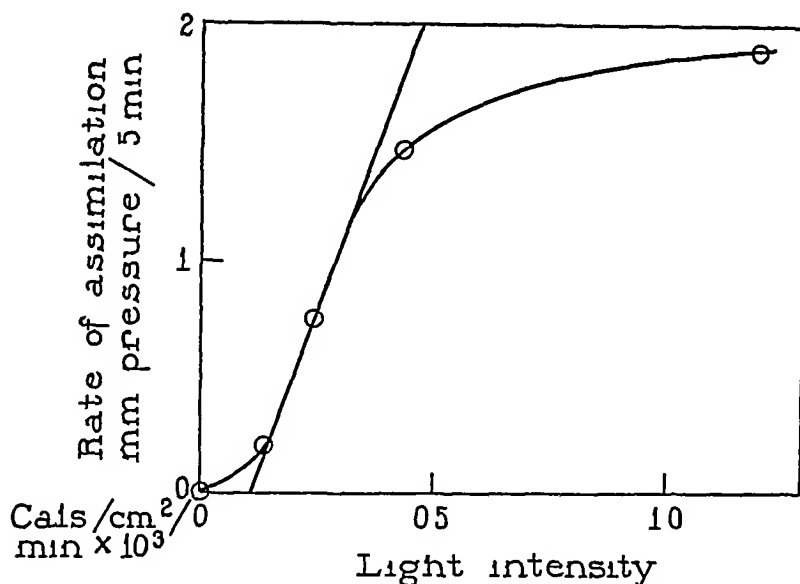
FIG 11 Rate of assimilation as a function of light intensity   Data of sample experiment   The intensity scale represents cals /cm ^{2}/min falling of the vessel bottom

TABLE VIII

Quantum Yield of Hydrogen + Carbon Dioxide Assimilation   Summary of Data

Fraction absorbed F	Quanta per H_2 γ	Intercept I_1 at $P = 0$ (extrap)	Previous treatment of cells
0 58	7 2	0 29	None
0 43	7 9	0 36	None
0 45	4 5	0 27	25° light, 5 hrs , 5 per cent CO_2/H_2
0 26	3 5	0 27	" " " " " " " "
0 45	2 9	0 28	25° light, 2 hrs , 5 per cent CO_2/H_2
0 36	8 1	0 39	Measurement in yeast extract growth medium
0 43	2 9	0 20	25° light, 1 hr , 5 per cent CO_2/ H_2
0 36	2 5	0 13	25° light, ½ hr , 5 per cent CO_2/Ar ½ hr , 5 per cent CO_2/H_2
0 34*	2 9	0 11	25° light, 1½hrs , 5 per cent CO_2/Ar 10 min , 5 per cent CO_2/H_2
0 17	2 2	0 05	23° dark, 1½ hrs , air

* Complete data for this experiment given at the end of the paper
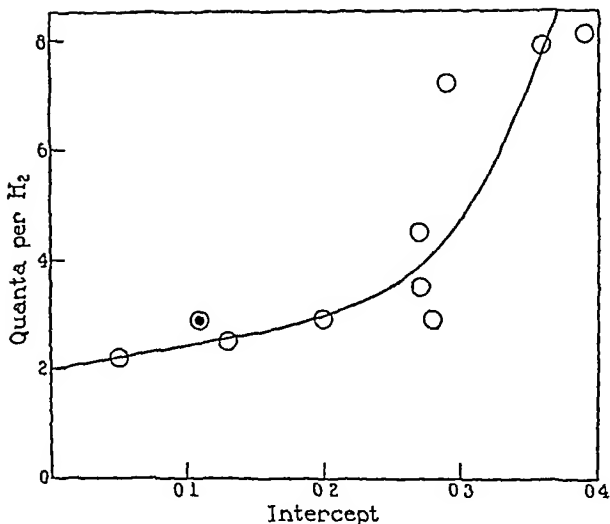
FIG 12 The data of Table VIII   The number of quanta required to assimilate one H_2, as calculated from the slope of the plots of rate of assimilation against light intensity, is shown as a function of the intercept of the extrapolated straight part of the curve on the intensity axis   Each point represents the results of one experiment   The point with the black dot is the sample experiment the data of which is given in full and plotted in Fig 11

This plot makes it evident that as the inhibiting effect seen at low light intensities is removed, the value approaches 2 quanta per H_2

We will designate by γ the number of mole quanta required to make 1 mole of H_2 disappear

$$\gamma = \frac{Quanta}{Molecules} = \frac{Einsteins}{moles} = \frac{cals \ per \ min}{c \ mm \ per \ min} \times \frac{2.24 \times 10^7}{33\,500}$$

$$\gamma = \frac{E}{P} \times 669$$

On the steepest part of the curves of rate of assimilation, h per 5 minutes against intensity, I_1, two points are taken and values of h and I_1 taken as differences

$$h = h_2 - h_1 \quad \text{and} \quad I_1 = (I_1)_2 - (I_1)_1$$

Rate of photosynthesis per minute then is

$$P = h \times \frac{K_{H_2}}{5} \text{ c mm per min}$$

and absorbed energy

$$E = I_1 \, q_1 \, F \text{ cals per min}$$

where q_1 is the bottom area of the bacterial suspension exposed to light and F is the fraction absorbed

In Table VIII we have summarized the quantitative experiments made as described above   As a measure of the amount of the flattening at the bottom of the curve we have taken the intercept of the straight line portion with the zero axis   This value, as well as the quantum yield, varies with the previous treatment of the bacteria, it appears to approach zero and the number of quanta 2 per H_2 as more favorable handling is approached   That is, 4 quanta are required for the reduction of one CO_2 molecule

DISCUSSION

The main point of this paper is to show that the mechanisms of photosynthesis in the green plant and in purple bacteria are similar because both types take about 4 quanta to reduce one CO_2 molecule Green plants fix energy by *making carbohydrate from CO_2 and H_2O* while in the case of a reaction with $2H_2$ and $1CO_2$ there is little if any energy change in going to carbohydrate or similar substances   Instead of reacting with water and splitting out O_2 the bacteria take in H_2

Green plants $H_2O + CO_2 \rightarrow (CH_2O) + O_2 - 114{,}000$ cals

Purple bacteria $2H_2 + CO_2 \rightarrow (CH_2O) ? + H_2O \pm 0$ cals
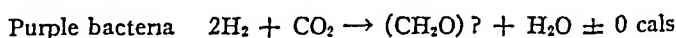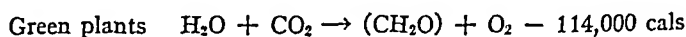
There is in these bacteria no reaction between H_2 and CO_2 in the dark as is the case in some species   The dark readings are very small and in the opposite direction, as though there were fermentation or acid production   Only with very dense cultures is this appreciable

In the quantum yield experiments the pressures were always negative There is no need for assuming a constant rate of a predominating dark reaction as in the case with green plants where respiration goes on faster than photosynthesis at the low intensities used for efficiency measurements As these experiments with bacteria were done anaerobically and no O_2 is produced there is no respiration Quantum yield experiments on CO_2 reduction by butyrate in *Spirillum rubrum* (which does not use H_2) were not very successful, possibly because of the high fermentation rate

It should be pointed out that the shape of the intensity curves cannot be attributed to a reaction dependent on having 2 or more quanta hit the same place nearly simultaneously because the curve is very much influenced by temperature and previous treatment of the bacteria As a working hypothesis not yet contradicted by any facts it is thought that at low light intensities another photochemical re action involving possibly CO_2 and an organic substance takes place in preference to the hydrogen assimilation With the hope of removing this hypothetical organic substrate the bacteria were treated in various ways as shown in Table VIII Shaking with air made the back extrapolated straight portion of the curve very nearly go through the origin and gave the highest efficiency Whatever the reason, this treatment considerably improved the yield Bacteria left in H_2 at $25°$ in 0 05 M $KHCO_3$ in the light for 24 hours were not motile nor photosynthetically active

The accuracy and number of the determinations is not yet as great as could be desired, but since the absorption measurements can be in error only in the direction corresponding to too large a number of quanta it seems fairly evident from Table VIII and Fig 12 that the probable value is 2 quanta per H_2 or 4 per CO_2

SUMMARY

1 The effect of H_2 tension, CO_2 tension, pH, time, light intensity, density of suspension, salt content of the medium, and certain spectral regions on the rate of photoassimilation of H_2 and CO_2 by *Streptococcus varians* has been studied

2 The method of making light absorption measurements with thin suspensions of bacteria is described

3 A light source, optical system, and filter for isolating 852 $m\mu$ with

894 mμ in sufficient intensity for photochemical work and an improved design of thermostat are given

4 The photoassimilation of $2H_2$ with $1CO_2$ apparently involves little over all energy change but nevertheless requires 4 quanta

It is a pleasure to thank Professor Warburg for laboratory accommodations and for his constant help and interest in the work   To all the workers in the Laboratory I am deeply indebted for suggestions, and particularly to Messrs Haas, Kubowitz, and Hartwig for much direct collaboration   Grateful acknowledgment is due to Professor van Niel who gave me the bacteria, taught me pure culture methods, and generously shared his stimulating ideas

Protocol

Sample Experiment

June 17, 1936

Streptococcus varians grown as described in the text

Time	Treatment
9 05–9 35	Centrifuged at 6,000 R P M
9 35	Suspended in 4 cc tap water, centrifuged 1 minute at 10,000 R P M to remove sediment Diluted about 50 times with 0 05 M KHCO₃ and shaken with argon 5 per cent CO₂, 7 0 cc put in manometer vessel
10 30–12 00	Put over show-case lamp at 25° with argon 5 per cent CO₂
12 00–12 10	Passed 5 per cent CO₂/H₂ through
12 20	Put at 9 2° in thermostat
1 03	Started readings
2 45	Experiment stopped

This time schedule is not typical   Generally centrifuging was done later in the morning and measurements made in the late afternoon

Absorption Measurements

Vessel thickness	Deflection without vessel	Deflection with vessel	$\dfrac{I}{I_0 \times 0\,93}$*
d cm	I_0	I	T
0 50	10 8	5 2	0 52
0 202	10 9	7 4	0 73
0 097	11 1	9 1	0 88

* Separately determined transmission of all vessels with either bleached suspension or water

Data Sheet of Sample Experiment
June 17, 1936

Temperature Beckman thermometer 3 02 = 9.3 C.	Time	Cathetometer readings		h	h Average

$I_1 = 1\,20 \times 10^{-2}$ cals./cm²/min

	min	*mm*	*mm*	*mm*	*mm*
3 02	0	4 19	22 23		
3 025	5	5 27 − 1 09	21 39 − 0 84	− 1 95	
3 025	5	6 30 − 1 03	20 55 − 0 84	− 1 87	1 88
3 00	5	7 25 − 0 95	19 74 − 0 81	− 1 76	

Neutral filter $C(T = 0\,360)$
$I_1 = 1\,20 \times 10^{-2} \times 0\,360 = 0\,43 \times 10^{-2}$ cals /min /cm²

3 22	0	8 41	18 71		
3 22	5	9 23 − 0 82	17 99 − 0 72	1 54	− 1 47
	5	9 97 − 0 74	17 32 − 0 67	1 41	

Neutral filters $B + C$
$I_1 = 1\,20 \times 10^{-2} \times 0\,360 \times 0\,567 = 0\,245 \times 10^{-2}$

3 01	0	11 30	16 12		
3 01	5	11 69 − 0 39	15 78 − 0 34	− 0 73	− 0 80
3 02	5	12 15 − 0 46	15 37 − 0 41	− 0 87	

$B' + C$
$I_1 = 0\,245 \times 10^{-2}$

3 01	0	12 88	14 70		
3 01	5	13 25 − 0 37	14 31 − 0 29	− 0 66	− 0 74
3 01	5	13 68 − 0 43	13 92 − 0 39	− 0 82	

$B + B' + C$
$I_1 = 1\,20 \times 10^{-2} \times 0\,575 \times 0\,567 \times 0\,360 = 0\,139 \times 10^{-2}$

3 02	0	13 84	13 78		
3 02	5	13 95 − 0 11	13 71 − 0 07	− 0 18	− 0 20
	5	14 06 − 0 11	13 61 − 0 10	− 0 21	

Dark
$I_1 = 0$

3 02	0	14 15	13 54		
3 02	5	14 20 − 0 05	13 49 − 0 05	− 0 10	
3 015	5	14 15 + 0 05	13 54 + 0 05	+ 0 10	0

At beginning

$\Omega = 322$ $I_1 = \dfrac{387 \times 10^{-2}}{322} = 1\,20 \times 10^{-2}$

After experiment

$\Omega = 318$

The average thickness of the suspension in the manometer is taken as $\frac{7\,0}{23\,8} = 0\,293$ cm   The transmission of this thickness was determined by graphical interpolation, plotting T against d, in this case it amounted to 0 66   Therefore,

$$F = (1 - 0\,66) = 0\,34 = \text{fraction of incident light absorbed}$$
$$\text{in manometer vessel}$$

$$T_w = 0\,96 \text{ for 1 cm water at 852 m}\mu$$

$$\begin{array}{ccc} First & & Second \\ window & Mirror & window \end{array}$$

$$R = 0\,92 \times 0\,95 \times 0\,96 = 0\,84$$

$$B = \left(\frac{7\,5}{2}\right)^2 \pi = 44\,4 \text{ cm}^2$$

$$I_1 = \frac{T_w R}{B} \times \frac{21{,}300}{\Omega} \times 10^{-3} = \frac{0\,96 \times 0\,84 \times 21{,}300 \times 10^{-3}}{\Omega\,44\,4}$$

$$= \frac{387}{\Omega} \times 10^{-3} \text{ cals /cm}^{-2}/\text{min}$$

This formula was used to calculate the intensities recorded in the data sheet and plotted in Fig 11 against the pressure readings   The slope of the steep part of the curve is $h/I_1 = 5\,5$ Therefore

$$E = I_1 \, q_1 \, F = 1 \times 10^{-3} \times 23\,8 \times 0\,34 = 8\,1 \times 10^{-3}$$

$$P = h \times \frac{K_{H_2}}{5} = 5\,5 \times \frac{1\,72}{5} = 1\,9$$

$$\gamma = \frac{E}{P} \times \frac{2\,24 \times 10^{-7}}{33{,}500} = \frac{8\,1 \times 10^{-3}}{1\,9} \times 669 = 2\,85 \text{ quanta per H}_2$$

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THE KINETICS OF PENETRATION

XIV The Penetration of Iodide into Valonia

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Former papers from this laboratory indicate that the penetration of a weak base, ammonia,[1] and of a strong base, guanidine,[2] is probably preceded by a chemical reaction with a constituent of the protoplasm On the other hand the penetration of a weak acid, hydrogen sulfide,[3] appears to take place by simple diffusion of molecular hydrogen sulfide through the non aqueous protoplasm

What is the method of penetration of a strong acid? The present paper is an attempt to throw light on this problem by an investigation of the rate of entrance of iodide into *Valonia macrophysa*, Kütz

The choice of hydrogen iodide for this investigation was a practical necessity, since of the other available acids, nitric[4] and hydrobromic, the former is already present in fairly large, but variable amounts, and the latter presents analytical difficulties in the presence of the high concentration of chloride ion in *Valonia*

Hydrogen iodide presents some theoretical difficulties owing to the possibility of oxidation or reduction in the cell   The reversible reaction, $I_2 + 2e \rightleftarrows 2I^-$, goes with the greatest ease in either direction according to conditions, while with much greater difficulty any free iodine produced may be oxidized to iodate   This will be discussed later

On the other hand, hydriodic acid is as strong an acid as hydrochloric, and with proper care it and its salts can be determined accu-

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[1] Osterhout, W J V , *Proc Nat Acad Sc*, 1935, 21, 125

[2] Jacques, A G *Proc Nat Acad Sc*, 1935, 21, 488

[3] Jacques, A G , *J Gen Physiol*, 1935–36, 19, 397

[4] Unpublished results indicate that the concentration of nitrate ion in fresh *Valonia* sap is between 0 02 and 0 01 molar

rately in very small amounts   Moreover with our method of analysis no iodide is detectable in normal *Valonia* sap

EXPERIMENTAL

The rate of entrance of iodide was studied from the standpoint of the effect of concentration, pH, and light   In most cases the cells were exposed to the modified sea waters in 125 ml bottles   Effective stirring was provided by means of stirrers described in previous papers [2] [5]

The modified sea waters were made up by adding appropriate quantities of 0 6 molar NaI solution   The pH was adjusted by adding either 0 6 N HCl or 0 6 N NaOH and aerating, as described previously,[6] and the pH of sea water and sap was determined by means of indicators and the Hellige double wedge colorimeter with the aid of calibration curves, as reported in another paper [7]   After exposure the cells were washed in ordinary sea water, rinsed in distilled water, and dried on filter paper   The sap was then extracted by means of a tuberculin syringe with a steel needle

The sap was then analyzed for iodide by oxidizing the iodide to free iodine and extracting the latter by chloroform   The concentration of iodine in the chloroform was then determined by means of the Zeiss-Pulfrich step-photometer, using the "S 53" or the "S 50" color filter [8]

In spite of the great amount of work which has been done on the determination of small amounts of iodine in biological materials, no standardized procedure which meets all objections has yet been worked out   For this reason it was considered necessary to develop for this investigation a method which would give accurate results for known amounts of iodide added to aqueous solutions resembling *Valonia* sap and to sap itself   Many of the objections to previous methods deal with the difficulty of incinerating the biological material in such a way as to avoid

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[5] Jacques, A G , and Osterhout, W J V , *J Gen Physiol* , 1929–30, **13**, 695

[6] Jacques, A G , and Osterhout, W J V , *J Gen Physiol* , 1933–34, **17**, 727

[7] Jacques, A G , and Osterhout, W J V , *J Gen Physiol* , 1931–32, **15**, 537

[8] Getman (Getman, F H , *J Am Chem Soc* , 1928, **50**, 2883) finds that the absorption maximum for 0 0005 M iodine in chloroform lies between 540 and 530 mμ Waentig (Waentig, P , *Z phys Chem* , 1910, **68**, 513) finds the absorption maximum at about the same point but his results were criticized by Ley and von Engelhardt (Ley, H , and von Engelhardt, K , *Z anorg Chem* , 1911, **72**, 55), who found that for 0 05 to 0 005 N solutions the maximum was at 500 mμ

Recently for solutions between 0 005 and 0 3 molar, Chatelet (Chatelet, M , *Ann chim* , Paris, 1934, **2**, series 11,5) has located the maximum at 508 mμ In our work with the Zeiss-Pulfrich spectrophotometer we have found about the same results with the light filters whose dominant wave lengths are 530 and 500 mμ ("S53" and "S50" in the manufacturers' nomenclature)   We have, however, found the latter more comfortable to use

loss of iodine But fortunately *Valonia* sap is sufficiently free of organic material to make incineration unnecessary However, questions have also been raised on the following points, (a) the best agent to employ to oxidize the iodide to free iodine particularly when other salts are present, (b) the solvent to be used to extract the free iodine, and (c) the effect of salts on the completeness of the extraction and on the titrimetric determination with Na S₂O₃.

With respect to (a), most authors seem to have preferred nitrous acid, as the oxidizing agent,[9] but KMnO₄ has been used [10] Our own experience with nitrite sulfuric acid mixtures and nitrite hydrochloric acid mixtures was disappointing, probably because of the presence of a fairly high concentration, 0 6 M of chloride in the sap For this reason we selected another oxidizer, KIO₃ This, of course, has been used extensively in macroanalysis but as far as we can ascertain not in micro work [11] The difficulty with this reaction which theoretically should go as follows,

$$5KI + KIO_3 + 6HCl \rightarrow 3H_2O + 6KCl + 3I_2$$
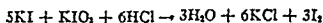
is that if the solution is strongly acid side reactions occur and part of the iodine from both the iodide and iodate appears as ICl, a compound which according to Philbrick,[12] is highly ionized in the presence of strong HCl, and is not extracted into carbon tetrachloride It is therefore necessary to prevent the formation of ICl, by keeping the hydrogen ion activity low This was done by using Richard's plan of acidifying with tartaric acid as recommended by Kolthoff [13]

It seems not improbable that the large excess of chloride in the sap may favor side reactions when iodides are oxidized by KIO₃ in the presence of strong acids The results given in Table I, dealing with the analysis of known iodide mixtures with and without the addition of sap salts, are significant in this connection In

[9] von Fellenberg T *Ergebn Physiol*, 1926, 25, 176 Reith, J F, *Rec trav chim*, 1929, 48, 254 Meerburg P A *Z phys Chem*, 1927 130, 105 Hercus C E, Benson, W N and Carter, C L, *J Hyg*, Cambridge England, 1925, 24, 321 These authors have also used a nitric sulfuric acid mixture as oxidizing agent McClendon (McClendon, J F *J Biol Chem* 1924, 60, 289) used nitrosyl sulfuric acid Allott, E N, Dauphinee, J A., and Hurtley, W H, *Biochem J* London, 1932, 26, 1665

[10] Bose, A C, and Bagchi K N, *Analyst* 1935 60, 80

[11] The reverse procedure whereby the iodide is transferred to iodate by a powerful oxidizing agent and is subsequently reduced by the addition of an excess of potassium iodide has, of course, been used extensively in determining very small amounts of iodide

[12] Philbrick, F A, *J Chem Soc*, 1930, 2254

[13] Kolthoff I M Volumetric analysis New York John Wiley and Sons, 1929, 2, 391 Kolthoff, I M, *Z anal Chem*, 1921 60, 403

these analyses each solution contained 0 0400 mg of iodine in the form of potassium iodide   Group 1 solutions contained the sap salts, KCl and NaCl, at the concentration found in *Valonia* sap, while Group 2 solutions were made up in distilled water   The iodide was oxidized to free iodine by the addition of KIO_3 and hydrochloric acid at the concentrations indicated at the heads of the vertical columns

In contrast with these results we found that when tartaric acid was used to acidify the oxidation mixture, the same result accurate to 5 per cent was obtained whether the sample was made up with sap salts or in distilled water   Furthermore the concentration of the tartaric acid seemed to be unimportant   In most of our determinations we estimate that it was between 0 01 and 0 05 normal

Our attempts to use nitrite with hydrochloric acid gave very much poorer results even than the iodate-hydrochloric acid mixtures with or without salts

TABLE I

Effect of Strong Acid on the Determination of 0 04 M Iodide by Oxidation to I_2 by KIO$_3$

	Group 1 Solutions made up with sap salts				Group 2 Solutions made up in distilled water			
Concn HCl, M	0 200	0 0400	0 0100	0 00333	0 200	0 0400	0 0100	0 00333
Iodine found, *mg*	0 0025	0 0092	0 0375	0 0417	0 0183	0 0417	0 0392	0 0408
				0 0417				0 0400
				0 0400				0 0400
			Av	0 04113			Av	0 04023

But when tartaric acid was substituted for the strong acid fairly satisfactory results were obtained   However, in view of the fact that iodate has the slight advantage that only 5/6 of the total iodine comes from the unbound iodide, it seemed desirable to retain it as the oxidizer

Other investigators have considered the effect of salts on the estimation of iodide   Thus Reith,[14] investigating von Fellenberg's colorimetric method in which the nitrite-sulfuric acid mixture is used as the oxidizer, found that large negative errors might occur in the presence of SO_4'', Cl', NO_2', Br', BrO_3', and ClO_3' Reith indeed concludes that the method is only good for pure solutions of iodide   Maljaroff and Matskiewitsch[15] have also had difficulty using the nitrite-hydrochloric acid mixture, but they have apparently attributed their losses to greater solubility of the iodine in the salt solutions, with consequent decrease in the partition coeffi-

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[14] Reith, J F , *Biochem Z* , Berlin, 1929, **216**, 249
[15] Maljaroff, K L , and Matskiewitsch, V B , *Mikrochemie*, 1933, **13**, 85

cient between the aqueous phase and the chloroform [16] To us this seems very unlikely for while it is true that the solubility of iodine increases slightly in NaCl solution, the effect is certainly too small to account for 50 per cent or even 100 per cent losses of iodine It seems much more probable that the failure of the method was due to side reactions

Weil and Sturm[17] also point out that they found low values for iodine when using von Fellenberg s method in the presence of large salt concentrations [18]

With respect to (b), Blum[19] has objected to the use of chloroform to extract iodine when the nitrite sulfuric acid mixture is used on the grounds that the color fades rather rapidly However he states that this fading does not occur when pure iodine is dissolved in chloroform Our experience has been somewhat similar to Blum's but we have found that the iodine-chloroform mixture extracted from a mixture with iodate tartaric acid as the oxidizer does not fade in 24 hours Moreover most of our determinations of the color (light absorption) of the extracts were made within 30 minutes of the extraction, so that errors from this source could scarcely occur

Settimj[20] has strongly recommended the use of CS_2 in place of $CHCl_3$ or CCl_4 on the grounds that at low concentrations the color with CS_2 is more intense, and that the distribution ratio strongly favors CS_2 There are, however, certain objections to CS_2 when the analysis is made by means of a spectrophotometer Thus it is clear from the results of Coblenz[21] and Waentig[22] that the extinction coefficient is not a linear function of the concentration of iodine, which behavior may possibly be connected with the fact that the distribution coefficient increases steadily with increasing concentration of iodine [*] It is inconvenient from the standpoint of the photometer because it does not permit the use of a linear curve

[16] In this connection, McClendon (McClendon, J F, *J Biol Chem* 1924 60, 289) has suggested that in order to obtain uniform distribution of the iodine between the phases the aqueous phase might be saturated with NaCl so that this salt might render negligible the effect of other salts which in most cases are present in much lower concentration

[17] Weil, W H , and Sturm A , *Deutsch Arch klin Med* , 1925 147, 166

[18] These authors prefer to check the colorimetric method by a supplementary titration method (in which the iodide is oxidized to iodate treated with an excess of KI and titrated with $Na_2S_2O_3$) when the amount of iodine involved is less than 3 γ In the present experiments however, the amounts were usually above 10 γ and never below 5 γ

[19] Blum, F *Schweiz med Woch* 1927, 57, 808

[20] Settimj M , *Ann chim applicata* 1927, 17, 432 (quoted from *Chem Abstr* , 1928 22, 654)

[21] Coblenz W M , *Phys Rev* , 1903, 16, 35

[22] Waentig, P , *Z phys Chem* , 1910, 68, 513

[*] See Jakowkin, A. A , *Z phys Chem* , 1895, 18, 585 1899, 29, 613

for the interpolation of results   With chloroform, on the other hand, the distri-
bution ratio appears to be fairly constant   At any rate it shows no trend   How-
ever, recently Chatelet[24] has asserted that while solutions of iodine in carbon
tetrachloride obey Beer's law those in chloroform do not   This leads him to the
conclusion that there are present in the chloroform solution two forms of iodine
One of these, however, disappears when the solution is diluted sufficiently   This
latter result is quite consistent with our own experience with iodine in chloroform
We have studied the concentration range from 0 1 mg per ml down to 0 005 mg
per ml with the Zeiss-Pulfrich spectrophotometer and have found that for the
dominant wave length λ = 500 mμ (filter "S 50") the solutions follow Beer's
law   A linear curve was therefore used in interpolating the results, and the size
of the samples was so chosen that the amounts of iodine to be extracted into the
chloroform fell between these limits

After consideration of all the factors involved the following analytical procedure
was adopted   Two equal samples of sap, usually colorless but occasionally colored
green by chloroplasts, were delivered into two 12 ml centrifuge tubes each of which
contained 2 ml of redistilled reagent grade chloroform   A drop each of 4 per cent
tartaric acid solution and 1 per cent potassium iodate solution were then added
to the test mixture   The second tube served as a blank   The test mixture was
then shaken gently to mix, and both tubes were capped and allowed to stand
2 to 5 minutes to complete the oxidation   They were then shaken energetically
for 30 seconds   In the blank, if the sap contained chloroplasts, the chloroform
was colored slightly green, while in the test solution under these conditions the
color was violet, modified slightly by the green color due to the extracted chlor-
ophyll   Both chloroform solutions were cloudy due to the inclusion of droplets
Both samples were centrifugalized at 1500 R P M for 3 to 5 minutes   The chloro-
form layers were then transferred by pipette to the absorption vessels   In order
to get a 50 mm layer for the determination of the light absorption with only 2 ml
of chloroform solution, micro vessels were used   The percentage absorption with
the "S 50" filter was then determined, several settings being made for each
determination and the vessels being interchanged to eliminate unequal lighting
of the two halves of the photometer   From the "per cent absorption" the
"extinction coefficient" was read off the table and the corresponding quantity of
iodine read off the calibration curve   In a good many cases no chlorophyll was
extracted so that the blank was colorless   In many others the rather feeble
absorption of chlorophyll in the green in comparison with the strong absorption
of the violet iodine made the error due to the chlorophyll negligible   It was only
in the case of very dilute iodine solutions that the compensation was needed
It is possible to avoid the need for the chlorophyll compensation by first centri-
fugalizing the sap to remove chloroplasts   And from time to time when extra
amounts of sap were available this was done, and the analyses made on chlorophyll-
free sap were compared with those made on the same sample with chlorophyll

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[24] Chatelet, M , *Ann chim* , Paris, 1934, series 11, 2, 5

present   The results were invariably the same within the limits of error of the method

The accuracy to be expected was determined by applying the method to known mixtures made up in artificial *Valonia* sap (i e a mixture of KCl NaCl at the average concentrations found in sap) and also to known iodine mixtures in distilled water   The results indicate that the error is of the order of ±4 per cent   Analyses on sap samples containing known amounts of iodide and varied amounts of chlorophyll indicated that the "chlorophyll compensation" method is valid, and finally that the error is of the order of ±4 0 per cent

RESULTS

Rate of Entrance at Constant pH —Table II and Fig 1 give the rate of entrance into the sap of *Valonia* of iodide from solutions with different concentrations of iodide at the normal pH of sea water   The

TABLE II

Rate of Iodide Pen tration into Valonia at pH 8 2

No	Concn iod de in S W	Concentration of iodide in sap at						
		24 hrs.	48 hrs.	72 hrs	120 hrs	200 hrs	320 hrs.	416 hrs.
	M	M	M	M	M	M	M	M
1	0 0400	0 00148	0 00240	0 00338	0 00490	0 00622	0 00328	0 00902
2	0 0200	0 000877	0 00125	0 00164	0 00263	0 00333	0 00435	0 00475
3	0 00280	0 000184	0 000251	0 000328	0 000480	0 000604	0 000831	0 000920

experiments were carried out in the rather feeble light of the laboratory and no change in pH due to photosynthesis was observed   Separate experiments under the same conditions indicated that growth was negligible   The curves have been drawn in this and other cases freehand to give an approximate fit   Each point on a curve represents the average of two or more analyses made on different samples of sap and, as usual, when large numbers of cells were available, cells of approximately the same size (0 2 to 0 4 ml) and shape were used   The cells used in any one experiment were from the same collection and had been seasoned in the laboratory (for 2 weeks or more) under the same conditions

pH Effect —Fig 2 shows that the external pH has little or no effect on the rate of entrance of iodide   As in previous experiments, the pH was adjusted by the addition of 0 6 N HCl or carbonate free 0 6 N
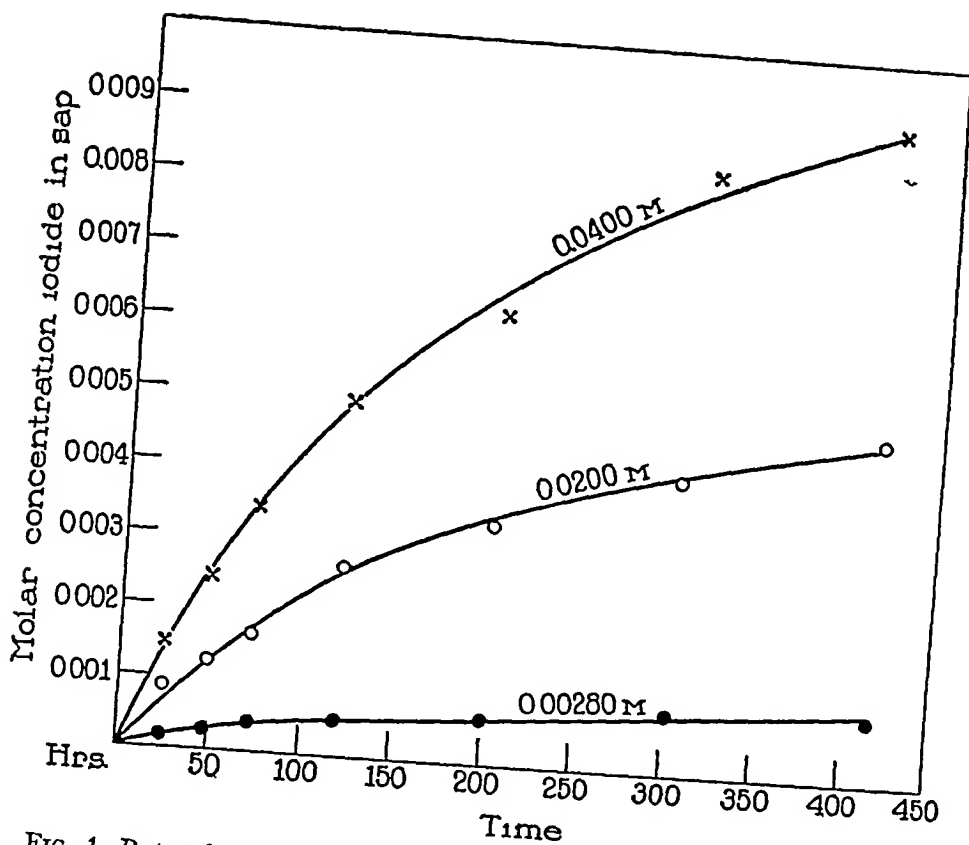
FIG 1 Rate of entrance, dx/dt, of iodide into *Valonia* at various external iodide concentrations
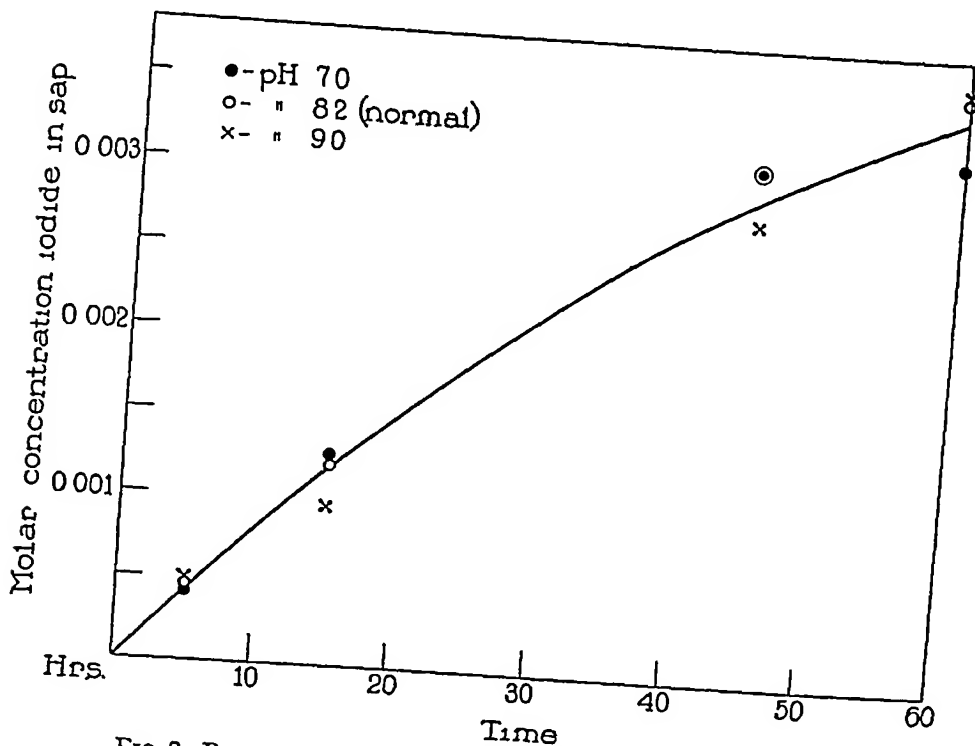
FIG 2 Rate of entrance of iodide into *Valonia* at various pH

NaOH, with aeration to bring the solutions into equilibrium with the CO_2 of the atmosphere   Some difficulty was experienced in keeping up the pH of the more alkaline solutions, probably because of the CO_2 production of the cells and absorption of CO_2 from the air, and to offset this the solutions were frequently renewed   Some falling off overnight, however, was inevitable [5]
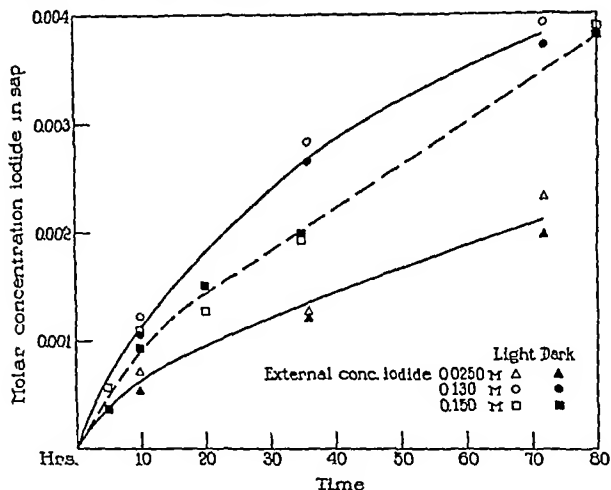
FIG 3   Rate of entrance of iodide into *Valonia* in darkness and illuminated

Light Effect—Fig 3 shows that light has little or no effect on the entrance of iodide   In these experiments the cells were exposed to the solutions in 125 cc bottles   The "dark" bottles were first covered with two coats of "black brushing lacquer," then with a layer of adhesive tape, and finally a coat of lacquer over the tape   The light

[5] The pH varied a little particularly at the high pH, but by changing the sea water frequently the effect of this was minimized   If there had been any evidence of a pH effect we should have resorted to flowing solutions as in previous experiments   But as there was not, this refinement seemed unnecessary

was provided by a 500 watt concentrated filament projection lamp, placed about 18 inches from the bottles   A half-inch layer of flowing water was interposed to absorb heat rays

TABLE III

Entrance of Iodide into Valonia macrophysa at Various External Concentrations of Iodide at pH 8 2

Exp	Molar concn of iodide in sea water	0 200	0 160	0 130	0 100	0 0800	0 0500	0 0200	0 0100
	Time		Molar concentration of iodide in sap						
	hrs								
1	10	0 00152	0 00116	0 00094	0 00085	0 00070	0 00054	0 00028	0 00020
	65	0 00496	0 00407	0 000338	0 00293	0 00246	0 00183	0 00116	0 00068
	Molar concn of iodide in sea water	0 0800	0 0580	0 0400	0 0200	0 0143	0 0072	0 0029	0 0014
2	20	0 00109	0 000808	0 000565	0 000275	0 000254	0 000145	0 000123	0 000098
	69	0 00295	0 00201	0 00120	0 000793	0 000670	0 000407	0 000236	0 000154
	Molar concn of iodide in sea water	0 0570	0 0400	0 0285	0 0200	0 0143	0 0072	0 0032	0 0014
3	20	0 00112	0 000883	0 000608	0 000392	0 000267	0 000133	0 000075	0 000028
	68	0 00380	0 00277	0 00212	0 00180	0 00125	0 000683	0 000410	0 000242
	88	0 00512	0 00377	0 00313	0 00210	0 00188	0 00114	0 000618	0 000315
	Molar concn of iodide in sea water	0 0400	0 0200	0 0143	0 0114	0 0086	0 0057	0 0029	0 0014
4	84	0 00233	0 00131	0 00107	0 00076	0 00066	0 00060	0 00033	0 00023
	156	0 00362	0 00219	0 00185	0 00158	0 00123	0 00093	0 00063	0 00035
	240	0 00485	0 00325	0 00223	0 00211	0 00173	0 00114	0 00087	0 00049

Effect of External Concentration —Table III and Fig 4 (*a, b, c,* and *d*) show the increase in rate as the external concentration increases   By means of several experiments, concentrations from 0 2 to 0 0014 M were tested

DISCUSSION

As Fig 2 shows, the rate of entrance of iodide is affected little or not at all by the external pH of the sea water between 7 and 9 This is in marked contrast with the entrance of H_2S, which has a "rate" [26] which is a linear function of the external concentration of undissociated H_2S and therefore of $f_\pm^2$ $[H^+]$ $[HS^-]$ [27] [28]

We may therefore conclude that entrance by diffusion as HI through the protoplasm is negligible It is true that a case might be made out for the hypothesis that the lack of pH effect is due to masking by other factors For example, if as a necessary preliminary to entrance a reversible reaction $HI + ZOH \rightleftharpoons H_2O + ZI$ must occur (where ZOH is a basic constituent of the protoplasm), the importance of the increase in the concentration of HI in accelerating the rate will become less as ZOH decreases in concentration, so that finally ZOH may be so limited in amount that any increase in the value of $[HI]$ or of f_H $[H^+] f_I-$ $[I^-]$ [9] becomes ineffective In sea water where the ionic strength is high compared with $[H^+]$ and $[I^-]$ as used in our experi ments we may regard the activity coefficients f_H and f_I as constant, and we may compare the effect of changing the product f_H $[H^+]$ $[I^-]$ (which is proportional to $[HI]$) by altering the pH, or by altering the iodide concentration

At pH 8 2, when the iodide concentration is 0 06 M, the product f_H $[H^+]$ $[I^-]$ is $10^{-9\ 42}$, and at pH 7 0 it is 10^{-8} [22] This change had no effect on the rate On the other hand in another experiment selected at random from the data of Table III, when the pH was 8 2

[26] 'Rate is used here to designate the function $\left(\dfrac{\partial x}{\partial a}\right)_t$ where x is the con centration of total sulfide in the sap and a the concentration of undissociated sulfide in the sea water

[27] Guggenheim (Guggenheim, E A , *J Phys Chem* 1930, 34, 1758) has shown that the single ionic activity coefficient has no physical significance We shall therefore replace it as far as possible by the mean activity coefficient $f_\pm$

[8] In this expression and hereafter square brackets refer to concentration in moles per liter, round brackets to activities in the same limits, and f is the mean activity coefficient when the concentration is expressed as moles/liter

[29] In this case it is convenient to speak of single ion activity coefficients, since the data available for calculation are the H ion activities which with reservations as to the liquid junction potential may be measured
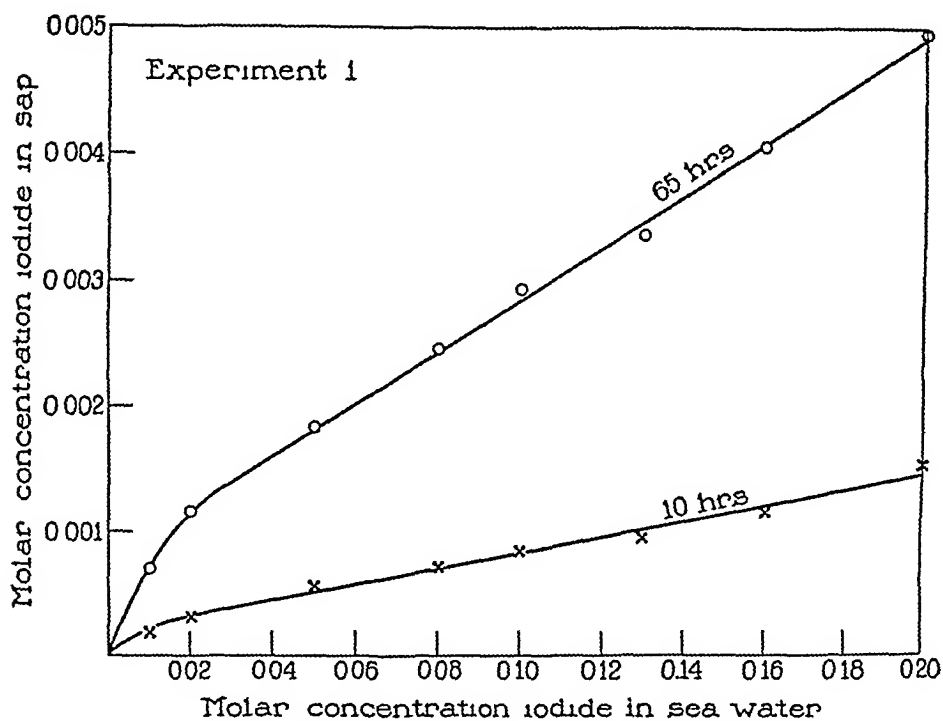
FIG. 4a

FIG 4, a, b, c, d Rate of increase of iodide in the sap plotted against concentrations of iodide in the external solution

FIG 4b

Fig 4c

Fig 4d

749
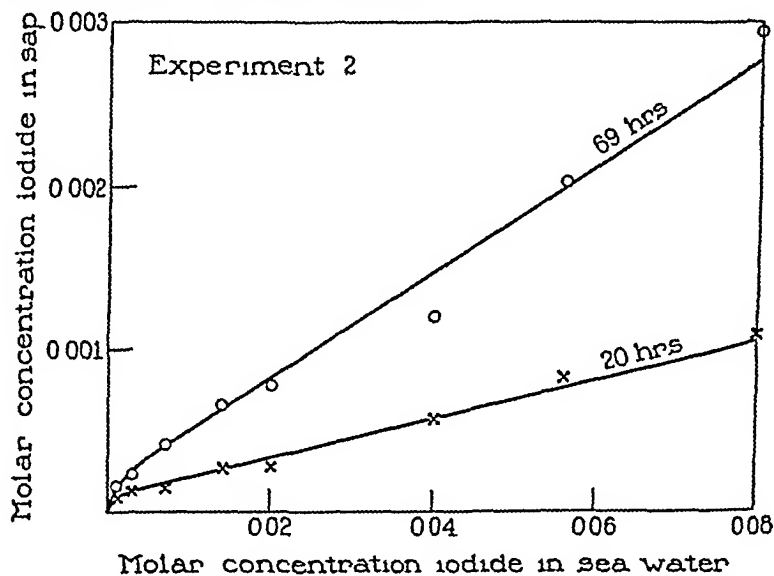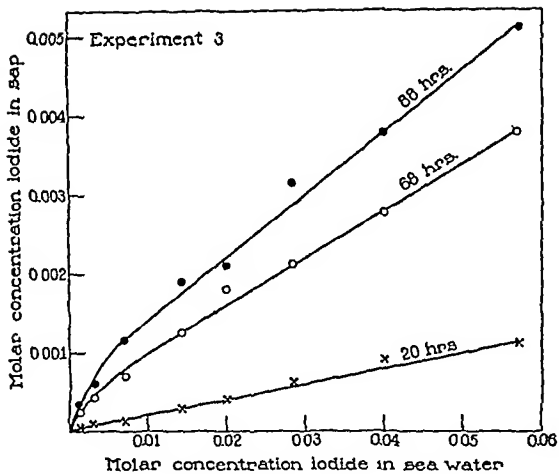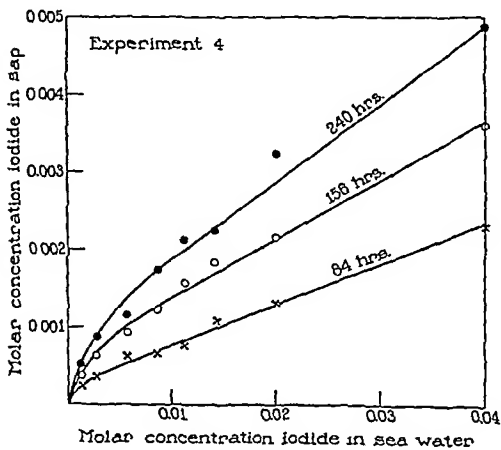
and the concentration of iodide 0 0580 M, the product was equal to $10^{-9\ 44}$, and when the concentration of iodide was 0 0400 M the product was equal to $10^{-9\ 40}$ This comparatively small change in the product had a pronounced effect on the rate   If the entrance were accompanied by combination with ZOH it should make no difference whether we increase the product by changing H^+ or by changing I^-

Further evidence that HI is not an important factor in the entrance of iodide is obtained from a consideration of the gradients   We may assume that the rate of increase of iodide concentration in the sap depends on the flux of iodide-containing solutes in the inner and outer non-aqueous protoplasmic surface layers   And since the same principles apply to both[30] for convenience we shall assume that there is only one layer and that the intervening watery layer may be neglected

When steady state diffusion is established (for entrance solely as HI) we may write under certain conditions that

$$\frac{dQ}{dt} = K'_{HI}\ ([H^+]_o\ [I^-]_o) - ([H^+]_i\ [I^-]_i)$$

where o and i refer to the sea water and sap, respectively, and Q is the quantity of HI which has passed into the sap in time t The derivation of this equation and the conditions involved will now be discussed

It can be shown[30] for the diffusion of a non-electrolyte that

$$vcf = D(fC_1 - fC_2)$$

where c is the concentration, v is the velocity of the solute, D is the diffusion constant, and C_1 and C_2 are respectively the concentrations of the solute at the boundaries of the diffusion layer   But $cv = Q$, where Q is the quantity of solute diffusing through a plane in unit time, whence

$$Qf = D(fC_1 - fC_2)$$

This in terms of HI diffusing as molecules in the non-aqueous protoplasm becomes

$$Q_{HI}\int_{HI}^{p} = D_{HI}\left(\int_{HI}^{o\,p} [HI]_{o\,p} - \int_{HI}^{i\,p} [HI]_{i\,p}\right)$$

where $o\,p$ and $i\,p$ refer to the sea water-protoplasm and sap-protoplasm interfaces and p to any plane in the protoplasm where the measurement is made   But

[30] Osterhout, W J V , J Gen Physiol , 1932–33, 16, 529

owing to the inevitable presence of unstirred layers at the interfaces, we may write for the sea water protoplasm interface

$$f_{HI}^{o.p.} [HI]_{o.p.} = S f_{HI}^{o} [HI]$$

and for the sap protoplasm interface

$$f_{HI}^{i.p.} [HI]_{i.p.} = S_i f_{HI}^{i} [HI]_i$$

where S is the partition coefficient and o and i refer to the sea water and sap respectively. Because of the stirring of the sap and sea water it may be assumed that the unstirred aqueous layers in contact with the protoplasm are at approximately the same concentration as the stirred aqueous solutions of which they are a part.

But

$$K S f_{HI}^{o} [HI] = S \left(f_{\pm}^{o} \right)^2 [H^+] [I^-]$$

and

$$K S_i f_{HI}^{i} [HI]_i = S_i \left(f_{\pm}^{i} \right)^2 [H^+]_i [I^-]_i$$

and substituting these values above we have

$$Q_{HI} f_{HI}^{p} = \frac{D_{HI}}{\lambda} \left(\left(S \left(f_{\pm}^{o} \right)^2 [H^+] [I^-] \right) - \left(S_i \left(f_{\pm}^{o} \right)^2 [H^+]_i [I^-]_i \right) \right)$$

In these equations under the experimental conditions f_{HI}^{p} the activity coefficient of HI in the non aqueous layer of the protoplasmic surface may be regarded as a constant [30] And since the ionic strength of the sap and sea water are so nearly alike (about 0 7 M) the mean activity coefficients $f_{\pm}$ and $f_{\pm}^{i}$ may also be regarded as equal and constant, and S and S_i, the partition coefficients, may also be regarded as equal and constant so that collecting all constant terms we have

$$Q_{HI} = K'_{HI} \left(([H^+] [I^-]) - ([H^+]_i [I^-]_i) \right)$$

or for diffusion with a gradient decreasing with time

$$\frac{dQ}{dt} = K'_{HI} \left(([H^+] [I^-]) - ([H^+]_i [I^-]_i) \right)$$

In this equation K'_{HI} may be called the "permeability constant" since it includes in addition to the diffusion constant of Fick's law, a variety of other terms which according to the above discussion may also be regarded as constants under the experimental conditions

In these experiments also, since the cells did not grow, and since $Q = VX$, where $V =$ volume and X is the concentration of iodide in the sap, we may write $K = K' - V$ and

$$\frac{dx}{dt} = K_{HI} \left([H^+]_o \, [I^-]_o - [H^+]_i \, [I^-]_i\right)$$

Unfortunately we cannot calculate either product from the available data, and we adopt the best possible expedient by using the equation in the form

$$\frac{dx}{dt} = K_{HI} \left(\int_{H^+}^{o} [H^+]_o \, [I^-]_o - \int_{H^+}^{i} [H^+]_i \, [I^-]_i \right)$$

which introduces the indefinite single ion activity coefficient of H^+ We do not have to know this value since we have from the measured pH of the sea water and sap the value of (H^+), which with reservations with regard to the liquid junction involved in the measurement is equal to f_{H^+} [H$^-$]

Fortunately this equation introduces practically no error since we are in all cases comparing these products in sap and sea water both of which have nearly the same ionic strength and hence the same value of f_{H^+}

From an experiment where $[I^-]_o = 0\ 06$ M, Fig 2, we derive the following values for the products $(H^+)_o \, [I^-]_o$

at pH 7 0, $10^{-8.22}$
at pH 8 2, $10^{-9.42}$
at pH 9 0, $10^{-10.22}$

The pH of the sap is approximately 6 0   Therefore when the external pH is 7 0 the gradient should vanish when $[I^-]_i$ becomes equal to 0 0060 M, at pH 8 2, when $[I^-]_i$ equals 0 00038 M, and at pH 9 0, when $[I^-]_i$ equals 0 00006 M   But actually at pH's 8 2 and 9, iodide continued to enter even after the limiting concentrations were reached, and further, the rates at all pH's were the same in spite of the great difference in the gradients

If the iodide moves into the sap by a process involving the product (H^-) [I$^-$], our experiments indicate that accumulation has taken place at pH 8 2 and 9 against a gradient   This can only happen if the cell itself supplies energy in some form capable of causing a predominantly

one way flow of the diffusing substance There is a tendency to consider this possible for the living cell because of its abundant supply of energy due to metabolism In the case of the entrance of Cl′, NO′₃, Br′, and I′ into *Nitella*, for example, Hoagland and Davis[11] have expressed the opinion that the energy of the cell metabolism is involved and that in some way light aids in the necessary transfer of energy, since the accumulation of these ions is greatly accelerated by light Our own experiments with the entrance of iodide into *Valonia* indicate that light is not an important factor, since we obtained no appreciable difference in rate between cells in total darkness and those strongly illuminated (Fig 3) In this case it seems unnecessary to refer the entrance directly to the cell metabolism since, as will be shown later, favorable gradients for the entrance of iodide exist, so that in all probability diffusional energy only is involved These will be discussed later

We now consider the nature of the function $\left(\dfrac{\partial x}{\partial t}\right)_a$ whose x and a are the concentrations of the diffusing substance in the sap and in the sea water For the case of simple diffusion of a molecular species in the protoplasmic surface layer, with equal partition coefficients S at both interfaces and a linear gradient, the equation

$$\left(\frac{\partial x}{\partial t}\right)_a = k \ (a - x)$$

where k is a constant which includes the partition coefficient, should apply Actually, in the case of the entrance of sulfide, where apparently only the diffusion of molecular H_2S is involved, the relationship did not hold instead the value of k diminished with time A number of reasons for this were suggested, and to these may now be added the possibility that diffusion gradients exist also in the sea water and in the sap These will be discussed later At the moment it is necessary to realize that the simple relationship can be applied only when a and x are known In the case of iodide we know the external and internal concentration of iodide at any time t, but we do not know the diffusing species in the protoplasm However, a calculation has been made, using for a and x the concentrations of iodide in the sea water and sap,

[11] Hoagland D R and Davis, A R, *J Gen Physiol* 1923–24, 6, 47

and the data of Table IV for the case when a was 0 04 M   The result shows that k is falling steadily with time

It is unlikely that the diffusing species in the non-aqueous protoplasmic surface is iodide ion   It is probably an undissociated molecule and we therefore consider the gradients of these   If the diffusing species is sodium iodide we may assume that the rate of its flux through the protoplasm or, what amounts to the same thing, the rate of gain of NaI by the sap, is proportional to the gradient $[NaI]_{o\ p} - [NaI]_{i\ p}$ where o p and i p represent the layers of protoplasm in immediate contact with the sea water and the sap respectively   The direction of the flow of NaI between sea water and sap will be determined (in

TABLE IV

Calculations Concerning Rate of Iodide Penetration at $[I^-]_o = 0\ 040$ M

Time	$[I^-]_i$	(1) $k_I = \dfrac{2\ 3}{t} \log \dfrac{[I^-]_o}{[I^-]_o - [I^-]_i}$	(2) $k_{NaI} = \dfrac{2\ 3}{t} \log \dfrac{[Na^+]_o [I^-]_o}{[Na^+]_o [I^-]_o - [Na^+]_i [I^-]_i}$
hrs			
24	0 00148	0 00160	0 000312
48	0 00240	0 00129	0 000250
72	0 00338	0 00122	0 000236
120	0 00490	0 00108	0 000209
200	0 00622	0 00084	0 000157
320	0 00828	0 00073	0 000132
416	0 00902	0 00061	0 000114

the absence of the supply of energy from the cell) by the activity product gradient $(Na^+)_o(I^-)_o - (Na^+)_i(I^-)_i$ But under the conditions set above for the diffusion of HI in the protoplasm we may write for the rate of NaI

$$\frac{d[I^-]_i}{dt} = K_{NaI} \left([Na^+]_o [I^-]_o - [Na^+]_i [I^-]_i\right)$$

which may be integrated to give

$$K_{NaI} = \frac{2\ 3}{t} \log \frac{[Na^+]_o [I^-]_o}{[Na^+]_o [I^-]_o - [Na^+]_i [I^-]_i} \tag{1}$$

The constant calculated on this basis was also found to decrease steadily with time (Table IV)

But if NaI can diffuse in the protoplasm as such it seems possible that KI can also $[K^+]_o = 0.012$ M in Bermuda sea water, and $[K^+]_i = 0.5$ M for the average cell, hence, assuming that the activity coefficients of $[K^+]_o$ and $[K^+]_i$ are equal in the experiment under discussion, where $[I^-] = 0.04$ M, when $[I^-]_i$ becomes 0.00096 M the concentration products are equal. Thereafter as $[I^-]_i$ increases, KI should come out of the cell. As Fig 1 shows the exit of KI should occur comparatively early in the process

For the situation when NaI is entering the cell and KI is emerging we may make the following calculation

$$\frac{d[NaI]_i}{dt} = k_1 ([Na^+] [I^-] - [Na^+]_i [I^-]_i) \qquad (2a)$$

and

$$\frac{d[KI]}{dt} = k_2 ([K^+] [I^-] - [K^+]_i [I^-]) \qquad (2b)$$

We now assume that all the iodide which enters or leaves the cell does so as NaI or KI

$$[Na^+]_o = 0.5 \text{ M in Bermuda sea water } [I^-] = 0.04 \text{ M}$$

$$\frac{d[NaI]_i}{dt} = k_1(0.02 - [Na^+]_i [I^-]_i) \qquad (2c)$$

$$\frac{d[KI]_i}{dt} = k_2(0.00048 - [K^+]_i [I^-]) \qquad (2d)$$

$$\frac{d[I^-]_i}{dt} = \frac{d([NaI]_i + [KI]_i)}{dt} =$$

$$0.02 \, k_1 + 0.00048 \, k_2 - (k_1 [Na^+]_i + k_2 [K^+]_i) [I^-]_i \qquad (2e)$$

In the sap, as NaI enters and KI leaves $[Na^+]_i$ must increase and $[K^+]_i$ decrease However, $\Delta[Na^+]_i$ and $\Delta[K^+]_i$ are small compared with $[Na^+]_i$ and $[K^+]_i$ and may be disregarded

In the average cell $[Na^+]_i = 0.1$ M Then

$$\frac{d[I^-]_i}{dt} = 0.02 \, k_1 + 0.00048 \, k_2 - (0.1 \, k_1 + 0.5 \, k_2) [I^-]_i \qquad (2f)$$

Integrating

$$\ln \frac{0.02 \, k_1 + 0.00048 \, k_2}{0.02 \, k_1 + 0.00048 \, k_2 - (0.1 \, k_1 + 0.5 \, k_2) [I^-]_i} = (0.1 \, k_1 + 0.5 \, k_2)t \qquad (2g)$$

'

Attempts to apply this equation by giving various values to k_1 and k_2 have proved to be unsuccessful   A pair of values which fit the upper part of the curve show large deviations at the lower part   This is scarcely surprising in view of the fact that the theory is oversimplified, particularly in neglecting the reversal of direction of movement of KI which should occur if this salt moves through the protoplasm at all

We now consider the "rate curve" defined as

$$\left(\frac{d[I^-]_i}{d[I^-]_o} \right)_t$$

Two possible diffusion processes will be considered

(a) Diffusion of sodium iodide alone leading to

$$\frac{d[I^-]_i}{dt} = k_{NaI} \left([Na^+]_o \, [I^-]_o - [Na^+]_i \, [I^-]_i \right)$$

which on integration gives

$$[I^-]_i = [Na^+]_o \, [I^-]_o \, (1 - e^{-k_{NaI}t}) \tag{3}$$

(b) Simultaneous diffusion of NaI and KI   Under the simplest assumption discussed above this leads to

$$\frac{d[I^-]_i}{dt} = k_1[Na^+]_o + k_2[K^+]_o \, [I]_o - k_1[Na^+]_i - k_2[K^+]_i \, [I^-]_i$$

Putting

$$k_1 \, [Na^+]_o + k_2 \, [K^+]_o = m \quad \text{and} \quad k_1 \, [Na^+]_i + k_2 \, [K^+]_i = n$$

on integration we get

$$[I^-]_i = \frac{m \, [I^-]_o}{n} \, (1 - e^{-nt}) \tag{4}$$

Each of these equations differentiated with respect to $[I^-]_i$ and $[I^-]_o$ at constant t gives

$$\left(\frac{\partial [I^-]_i}{\partial [I^-]_o} \right)_t = \text{const} *$$

* It is assumed, of course, that the concentrations of K and Na do not change seriously in either sap or sea water

In the penetration of H_2S the relationship

$$\frac{\partial [H_2S]_i}{\partial [H_2S]_o} = \text{const}$$

was true   But in the present case the relationship does not appear to
hold at lower concentrations   However, after a certain concentration
of $[I^-]_o$ is passed each curve becomes linear   The concentration at
which this break occurs is not the same in all cases, but in general we
may say that it lies between an external concentration of 0 01 and
0 02 molar

Several ways of considering the observed facts suggest themselves
as follows   (a) The entrance of iodide takes place by diffusion of a
molecular species in the protoplasm but part of the iodide in the cell is
immobilized by combination with a constituent of the sap which is
present in limited amount   Under these conditions the rate of en
trance will be favored more at lower concentrations of I_o, since a greater
portion of the entrance will take place according to the equation

$$\frac{d[I^-]_f}{dt} = k$$

i e , without any back pressure   However, this explanation is unten
able in the present case for, as reference to Fig 4 shows, the points of
inflection of the curves in a single experiment do not show any tend
ency to lie on a line parallel to the axis of abscissae, as would be re
quired by the immobilization hypothesis

(b) The entrance of iodide is by diffusion of molecular species in
the protoplasm but in addition to the gradients of the diffusing sub
stances in the protoplasm there are also gradients in the aqueous
solutions in contact with the protoplasm   The effect of these diffusion
gradients has been studied by Lewis and others[32] for the absorption of
gases and by Roughton[33] for the uptake of O_2 by blood corpuscles
The cell and its surroundings include the following regions, the sea
water, the cellulose envelope, the protoplasm, and the sap   Fig 5
shows schematically the possible unstirred regions in which concentra
tion gradients may exist

For the sake of simplicity Regions II and III in Fig 5 are considered
as one layer [34]   Since in the case under discussion the sea water was

[32] Lewis, W K and others, Absorption symposium, *Ind and Eng Chem* ,
1924 16, 1215

[33] Roughton F J W , *Proc Roy Soc London Series B*, 1932 111, 1

[34] There are practical grounds for this   since all the bioelectric experiments on
Valonia and *Nitella* indicate that the envelope is as permeable to electrolytes as
the aqueous solution   Thus when a cell is transferred from one solution to another
the P.D associated with its new environment is established within a few seconds

stirred the thickness of Region II may be very small and hence the diffusion gradient in it may be negligible   Region III, however, can scarcely be stirred under any conditions   Similarly we have no control over the amount of stirring in the protoplasm so that the thickness of IV and VI are uncontrolled [35]   Bioelectric measurements indicate[36] that these two layers are dissimilar   Finally the thickness of Region VII depends on the amount of stirring in the sap   This
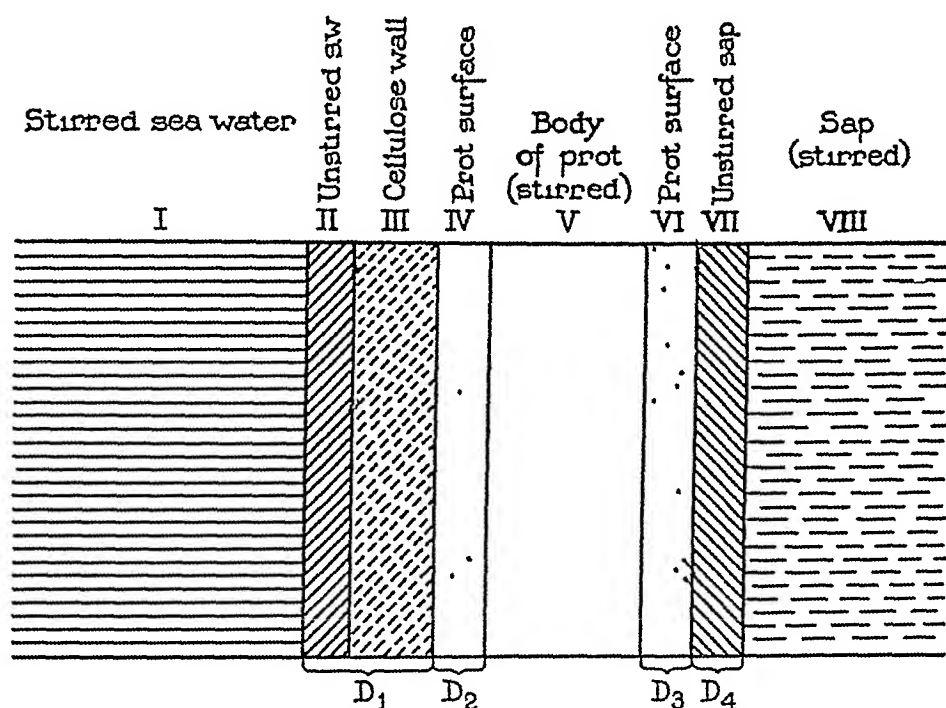
FIG 5 Schematic representation of diffusion layers involved in iodide entrance

depends on the stirring secured by the rolling of the cells as they fall through the sea water in the bottles, which was sufficient to keep in

[35] The picture of the protoplasm which best fits the numerous bioelectric measurements of Osterhout and his coworkers (Osterhout, W J V, *Ergebn Physiol* , 1933, 35, 967) consists of at least two dissimilar non-aqueous layers between which lies an aqueous layer   This might mean the introduction of more regions between IV and V, and V and VI, but inasmuch as the introduction of new layers does not alter matters essentially they are disregarded

[36] Osterhout, W J V, Damon, E B , and Jacques, A G , *J Gen Physiol* , 1927–28, 11, 193

brisk motion random dark particles occasionally seen in the vacuoles, and on the stirring due to convection   This may be considerable   Nevertheless it is not inconceivable that the thickness of VII is appreciable and that a concentration gradient exists in it

Jacobs[37] has indicated the method of deriving equations for the rate of flow $\dfrac{dQ}{dt}$ in the steady state of a solute through a series of dissimilar layers

According to Fig 5 we have in the present case four possible layers, II–III, IV, VI, and VII   Because V is adequately stirred, IV and VI are considered to be adjacent layers there being no concentration gradient in V   Moreover the customary assumption is made that the layers are so thin that the time for the establishment of steady state conditions is an inappreciable fraction of the total diffusion time

Let the diffusion layers in the cell be D_1 D_2, D_3, D_4 the layer thicknesses h_1, h_2, h_3 h_4 and the partition coefficients be S_1, S_2, S_3 and S_4[28] and a and x the concentrations of the diffusing solute in the sea water and the sap   Then

$$\frac{dQ}{dt} = \frac{S_1 D_1 S_2 D_2 S_3 D_3 S_4 D_4 \,(a - x)}{h_1 S_2 D_2 S_3 D_3 S_4 D_4 + h_2 S_1 D_1 S_3 D_3 S_4 D_4 + h_3 S_1 D_1 S_2 D_2 S_4 D_4 + h_4 S_1 D_1 S_2 D_2 S_3 D_3} \quad (5)$$

This shows that the form of the diffusion curve is independent of the number of layers even when the thicknesses, the diffusion constants, and the partition coefficients are different $\left(\dfrac{\partial x}{\partial a}\right)_t$ remains constant   Hence if the entrance of I⁻ takes place by diffusion under steady state conditions, the presence of diffusion gradients in several dissimilar layers cannot account for the observed shape of the curve where I is plotted against I₀

If, however, the sap is completely unstirred, the vacuole becomes part of the diffusion layer VII   Up to now we have assumed the total thickness of the diffusion system to be so small that the areas of the inner side of VII and the outer side of II–III are equal   This is not

[27] Jacobs M H Ergebn Biol 1935 12, 1
[28] The partition coefficients are as follows S_1 between Regions I and II + III (Fig 5) S_2 between II + III and IV S_3 between IV and VI, S_4 between VI and VII   Region V is, for convenience regarded as absent S_1 and S_3 are equal to 1 and S_2 might be equal to S_4 Moreover D_1 might equal D_4 but we put the equation in its most general form

true because diffusion is taking place into an enclosed space, but when the sap is adequately stirred we may deal with the diffusion as if it were taking place in a volume bounded by plane parallel surfaces When the vacuole is wholly unstirred it is necessary to take the shape of the cell into account   The natural cell is unsymmetrical but for convenience it may be regarded as a sphere[39] with the diffusion region extending in a series of concentric layers from the stirred mass of the sea water to the center of the vacuole

For a homogeneous spherical system Jacobs has derived the equation

$$\frac{x}{a} = 1 - \frac{6}{\pi^2}\left(e^{-\frac{\pi^2 D t}{R^2}} + 1/4\, e^{-\frac{4\pi^2 D t}{R^2}} + \qquad\right) \qquad (6)$$

where a is the constant concentration at the surface of the sphere, x is the average concentration in the sphere, and R is the radius of the sphere   The cell is not homogeneous but as has been shown the presence of several dissimilar layers affects the rate of diffusion but not the form of the diffusion equation

In this case also t is an exponential function of x and $\left(\dfrac{\partial x}{\partial a}\right)_t = $ const

Under unstirred conditions, therefore, for a single cell the form of the curve, when x is plotted against t or against a, is unchanged   But in our experiments the vacuole was stirred so that the problem in our case becomes roughly that of diffusion through a thin closed curved membrane of spherical shape   As Fig 5 shows, this is probably composed of several layers but, as Equation 5 (p 759) indicates, this does not affect the form of the diffusion equation   Moreover if in Equation 5 one of the terms $S_n D_n$ is much smaller than any of the others it will obviously control the rate and the diffusion system can be treated as homogeneous   The external concentration of the diffusing solute is a and the internal concentration in the vacuole at time $t = x$, and if the concentration gradient is linear so that $\dfrac{dQ}{dt}$ is a constant, we may write

$$dQ = V_1\, dx = \frac{D'\, 4\pi r_1 r_2\, (a - x)}{r_2 - r_1}\, dt^{**} \qquad (7)$$

** *Cf* Jacobs, M H , *Ergebn   Biol* , 1935, **12,** 125

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[39] Some cells are rather more cylindrical but the same principles apply

where r_1 and r are the radii of the inside and outside respectively of the spherical shell and V_1 is the volume of the stirred portion of the system in the vacuole It should be noted that D' is not the D of Fick's equation but is a "permeability constant" including diffusion constants in the protoplasm, partition coefficients, etc

On integration

$$\ln \frac{a}{a-x} = \frac{D}{V_1} \frac{4\pi\, r_1 r \cdot t}{(r_2 - r_1)} = \frac{3D'\, r_1 t}{r_1{}^2 (r_2 - r_1)} \tag{8}$$

But r_2 and r_1 are nearly equal Hence, as the equation shows, the time required for any degree of "saturation" of the sap is directly proportional to the radius of the cell and the thickness of the diffusion region This gives a mathematical basis for the well authenticated fact that the rate of diffusion into dead cells of unequal sizes is not "exponential "[5]

From the data of Table IV we can calculate by the use of the above equation values for D' for cells of various sizes and with different thicknesses of the diffusion layer [40] Under the following conditions $a = [Na^+] [I^-] = 0\ 02, x = [Na^+]_i [I^-]_i = 0\ 00020, t = 13, r_1 = 0\ 25$ cm , and $r_2 = 0\ 2505$ corresponding to a thickness of 5 microns, $D = 3\ 22 \times 10^{-8}$ cm ^{2}/hr If $r_2 = 0\ 255$ corresponding to a thickness of 50 microns, $D' = 3\ 1 \times 10^{-7}$ cm /hr With the other conditions the same and $r_1 = 0\ 5$ cm , $r_2 = 0\ 5005$ cm , $D' = 6\ 4 \times 10^{-8}$ cm /hr [41]

None of the cells used in the experiment was over 0 50 cm or less than 0 25 cm in radius The thickness of the protoplasm was less than 10 microns [4] For this reason the extremely low value of D', the permeability constant, is probably significant, for the diffusion constant of NaI in water at 0 04 M is of the order of 0 03 cm ^{2}/hr [43] Assuming

[40] Since in the experiment the rate of entrance of iodide took place with a falling value for the ' monomolecular ' constant, we have selected for the calculation values at the beginning of the experiment when the falling off was proportionally less

[41] In case the vacuole is not stirred $r_2 - r_1$ becomes greater and this would affect the value of D

[4] Doyle's results with *Valonia macrophysa* of Tortugas indicate that the protoplasm is from 5 to 8 μ thick Doyle W L *Papers from the Tortugas Laboratory of the Carnegie Institution* 1936 29, 13

[43] Landolt H , and Börnstein R , Physikalisch-chemische Tabellen, Berlin, Julius Springer 5th edition, 1923, 1, 247

that this value in water corresponds to the permeability constant of NaI in the living cell it is clear that its entrance into the cell is impeded very greatly   The impediment must be either in the cellulose wall, or in the protoplasm   But the cellulose wall when wet with sea water appears to be so permeable that diffusion in it is as rapid as in water   Thus, as Blinks[44] found, a freshly killed cell of *Valonia ventricosa*, a closely related form, has the low resistance corresponding to the specific resistance of the extracted sap   The protoplasm must therefore be responsible for the slow entrance of iodide

In these calculations the cell is considered to be a sphere but other shapes give similar results [45]

We may sum up by saying that if we take the value of D' to be of the order of 10^{-8} the passage of NaI through the protoplasm is about a million times as slow as it would be through water   The protoplasm is almost impermeable[46] to $MgSO_4$, $CaSO_4$, and CsCl Since the protoplasm is mostly water we may suppose that the hindrance to the passage of these substances lies almost entirely in the non-aqueous surface layers   It does not seem probable that they could act thus if they were unimolecular

An interesting question now arises in connection with the penetration of H_2S which was studied previously [3]   In that case the rate of entrance was very high compared with that of iodide   Assuming, however, that the penetration involved only diffusion, use of data in

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[44] Blinks, L R , *J Gen Physiol* , 1929–30, 13, 361

[45] A large number of cells are roughly prolate spheroids   The corresponding equation for the calculating of the permeability constant is

$$\ln \frac{a}{a - x} = \frac{tD}{H} \left(\frac{3}{2a} + \frac{3}{2eb} \arcsin e \right)$$

where H is the thickness of the diffusion layer, a and b are respectively the major and minor semi-axes of the ellipse which generates the solid, and e is its eccentricity $= \dfrac{\sqrt{a^2 - b^2}}{a}$ Taking an average cell $a = 0\ 700$ cm , $b = 0\ 255$ cm , and $H = 0\ 0005$ cm which has almost the same volume as the sphere of radius $0\ 5$ cm $D = 3\ 58 \times 10^{-8}$ For the same volume of sap the calculated value of D will be inversely proportional to the area of the surface which encloses it

[46] Osterhout, W J V , *Ergebn Physiol* , 1933, 35, 981 (Table I), also Cooper, W C , Jr , Dorcas, M J , and Osterhout, W J V , *J Gen Physiol* , 1928–29, 12, 427

Table II of the H_2S paper gives $a = 0\ 00625$, $x = 0\ 0009$, $t = 1$ min ute   If we put $r_2 - r_1 = 0\ 0005$ cm , and $r_1 = 0\ 5$ cm in Equation 8 (p 761) we arrive at $1\ 29 \times 10^{-5}$ cm²/min , as the value of the per meability constant D' But the diffusion constant of H_2S in water[47] is of the order of $1\ 0 \times 10^{-3}$ cm²/min   From these results we may be confident that in the penetration of H_2S also the protoplasmic layer is the deciding factor [48]

A calculation for CO_2 leads to similar results   In this case $D' = 1\ 46 \times 10^{-6}$ cm /min and D for the diffusion of CO_2 in sea water is of the order of $1\ 0 \times 10^{-3}$ cm²/min   We conclude therefore that in this case also the protoplasm controls the rate of entrance

It is interesting to find that Collander and Bärlund[49] have come to similar conclusions for the penetration of certain non-electrolytes into the cylindrical cells of *Chara ceratophylla*   They have compared the times required to half saturate the sap of dead cells with the calcu lated time required to half saturate "by diffusion" cylinders of the same dimensions as the cells   The two sets of figures agreed fairly well in that the actual time was about 4 times the theoretical time so that some impediment is offered by the cell wall   However, the times for half saturation in living cells were with two exceptions[50] 70 to more than 50,000 times the theoretical

It is evident that H_2S enters *Valonia* at a rate enormously greater than that of NaI (according to the figures just given it enters more than 10,000 times as fast)   Although permeability to H_2S and to CO_2 appears to be much greater than to NaI (and to HI) it might be misleading to generalize this by saying that weak electrolytes always enter more rapidly for this idea is contradicted by experiments with models which show that a strong electrolyte with a high partition coefficient may pass through a non aqueous layer more rapidly than a weak electrolyte or a non electrolyte with a lower partition coefficient

[47] Landolt, H , and Börnstein, R , Physikalisch chemische Tabellen Berlin, Julius Springer, 5th edition 1923, 1, 248

[48] In view of this we need not raise the question whether H_2S is more soluble in the non aqueous layer than in the aqueous part of the protoplasm

[49] Collander, R , and Bärlund, H , *Acta bot fenn* , 1933, 11, 1

[50] Methyl alcohol and trimethyl citrate penetrated almost as rapidly into living cells as into dead cells

But a weak acid or base may be expected to penetrate more rapidly than its salt [51]

So far it has been assumed that steady state (linear gradient) diffusion between the sea water and sap is established at once   But obviously it requires finite time   Without knowing the value of the diffusion constant for NaI in the protoplasm we cannot evaluate the time required for the establishment of linear gradient diffusion by the recognized methods [52]

The time required for approximate attainment of the steady state is given by the relationship

$$t = \frac{H^2}{\pi^2 D} \ln \frac{2}{\epsilon}$$

where ϵ is the fraction by which the system varies from the steady state

Putting H = 10 microns and D' = 0 0004 cm^2 per hour and ϵ = 0 0001, i e 1/100 of D in aqueous solutions

$$t = 9 \text{ seconds}$$

Thus the time required to establish the steady state is so brief, even when the absolute rate of entrance is very slow, as to be negligible,[53] and can have no bearing on the observed falling off in the value of k for NaI penetration or the deviation of the $\left(\dfrac{\partial i}{\partial a}\right)_t$ curve from the linear form

(c) Finally the shape of the $\left(\dfrac{\partial i}{\partial a}\right)_t$ curve inevitably suggests that two processes are involved in iodide entrance   For example, as Fig 6 shows, the iodide may be entering in part by diffusion, a process which gives a linear relationship between i and a (Curve I) and in part by a process which becomes less effective with time (Curve II)   The

[51] Osterhout, W J V , *Bot Rev* , 1936, 2, 303 (footnote 27)

[52] *Cf* Jacobs, M H , *Ergebn Biol* , 1935, 12, 62

[53] It is not necessary to take into account the partition coefficient in calculating the time for the attainment of steady state diffusion since S is only concerned with the concentration of the diffusing substance in the unstirred protoplasmic layers in contact with the sea water and sap, and these terms do not enter into the equation

combination yields Curve III which is of the shape found   We can only speculate as to the nature of the process responsible for Curve II Osterhout has shown that for the entrance of ammonia into *Valonia* a curve which has the required shape is obtained and that this is plausibly explained by assuming that a reversible reaction between NH_4OH and $H\lambda$, an acidic constituent of the protoplasm which is present in limited amounts, precedes penetration   In the present case, however, it is difficult to see what the reaction can be, for as has been pointed
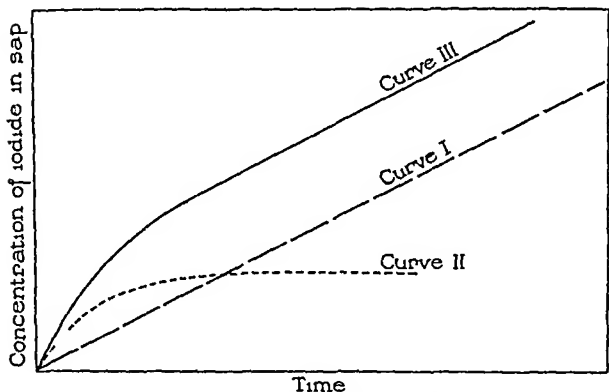
FIG 6 Hypothetical curves for iodide entrance Curve I, entrance by diffusion Curve II, entrance by combination Curve III, the resultant of the two processes

out above, there is very little reason to assume that a reaction between HI and a basic constituent of the protoplasm can occur   The nature of the reaction if it takes place is therefore entirely unknown

SUMMARY

When 0 1 M NaI is added to the sea water surrounding *Valonia* iodide appears in the sap, presumably entering as NaI, KI, and HI As the rate of entrance is not affected by changes in the external pH we conclude that the rate of entrance of HI is negligible in comparison

with that of NaI, whose concentration is about 10^7 times that of HI (the entrance of KI may be neglected for reasons stated)

This is in marked contrast with the behavior of sulfide which enters chiefly as H_2S It would seem that permeability to H_2S is enormously greater than to Na_2S Similar considerations apply to CO_2 In this respect the situation differs greatly from that found with iodide

NaI enters because its activity is greater outside than inside so that no energy need be supplied by the cell

The rate of entrance (*i e* the amount of iodide entering the sap in a given time) is proportional to the external concentration of iodide, or to the external product $[N^+]_o[I^-]_o$, after a certain external concentration of iodide has been reached   At lower concentrations the rate is relatively rapid   The reasons for this are discussed

The rate of passage of NaI through protoplasm is about a million times slower than through water   As the protoplasm is mostly water we may suppose that the delay is due chiefly to the non-aqueous protoplasmic surface layers   It would seem that these must be more than one molecule thick to bring this about

There is no great difference between the rate of entrance in the dark and in the light

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REACTIONS OF LIMULUS TO ILLUMINATED FIELDS OF DIFFERENT AREA AND FLICKER FREQUENCY

By ERNST WOLF and GERTRUD ZERRAHN WOLF

(From the Marine Biological Laboratory, Woods Hole, and the Biological Laboratories, Harvard University, Cambridge)

For the reaction of various Arthropods to stimulation by intermittent light, the relation between illumination and critical flicker frequency has been well established (Salzle (1932), Wolf (1933-34), Crozier, Wolf, and Zerrahn Wolf (1936-37a, b), Brocker, (1935)) Intermittent stimulation at flicker frequencies *below* fusion produces an increased reactiveness compared with steady light This was found for several insects (Mast and Dolley (1924, 1925), Wolf (1933), Zerrahn (1933)) It seems that under such conditions reaction depends upon the frequency and number of transitions of the retinal elements from one state of excitation into another (Wolf and Crozier (1932-33)) For vertebrates a pronounced effect of slow flicker as evidenced by the migration of retinal pigments has recently been described (Arey and Price (1936)) To obtain information about the effect of seen areas of different dimensions, illumination, and flicker frequency, and to establish quantitative relations, the honey bee has been used (Wolf and Zerrahn Wolf (1934-35)) For the bee as a fast moving insect, the strong reaction to intermittent stimulation seems of great importance (Wolf and Zerrahn Wolf (1936-37)) If flicker of low frequency was generally a strong stimulus for Arthropods, relations such as found with the bee should be found in other forms as well In this study we used *Limulus* *Limulus* has been proved to give rather precise phototropic responses (Loeb (1893), Northrop and Loeb (1922-23), Cole (1922-23)) At the same time we possess a good deal of information about the action of the optic nerve fibers in *Limulus* (Graham (1932), Hartline (1929-30), Hartline and Graham (1932))

With the help of Fig 1 the experimental arrangement can be understood   In a vertical piece of ply-wood two openings 17 7 × 17 7 cm are cut   The distance between the centers of the two holes is 32 cm   Into each opening opal screens are fitted which are illuminated from behind   The areas of the illuminated fields can be varied by means of cardboard frames, reducing the original area The ratios and areas of the test fields used are given in Table I   The frames
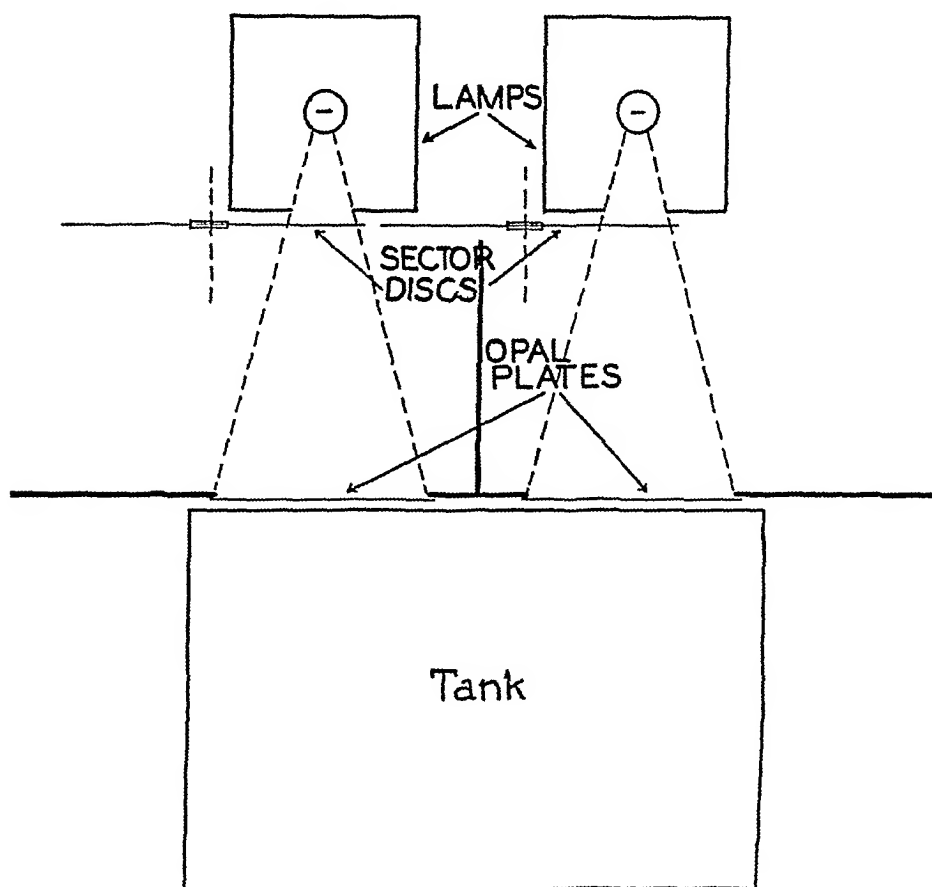
Fig 1 Apparatus for testing the reaction of *Limulus* to two illuminated fields differing in area and in flicker frequency

are made so that their lower edges coincide with the lower edge of the opening in the ply-wood, the illuminated fields are thus at "eye level" of our animals

In front of the wooden wall is a tank 64 × 45 cm filled with sea water as high as the upper margin of the illuminated fields   The bottom of the tank is slate   Since the experiments are performed in a dark room, influences by outside light or reflections from the walls are minimal

The opal screens are each illuminated by a 200 watt concentrated filament lamp. The light sources are set back 75 cm from the screens to secure an even illumination. The brightness of the lamps is determined and adjusted in such a way that the illuminations of both fields are equal. For intermittent stimulation by light, sector discs can be inserted in front of the openings of the lamp houses. The axles for the discs are connected by a gear and chain drive which permits keeping the same flicker ratio of the two fields at any desired flicker frequency. The sector wheels are cut so that with every revolution 180° permits light to pass through and 180° leaves the animals in darkness. The open half circle can be split into sectors $1 \times 180°$, $2 \times 90°$, $3 \times 60°$, 4×45, and $5 \times 36°$. The discs are driven by a 1800 R.P M , D C. motor, the speed of which can be controlled by a rheostat. Between the motor and the sector wheels a reduction gear box is inserted so that, by adjusting the rheostat, flicker fre

TABLE I

Field Sizes Used for Comparison of Effects of Areas of Flickered Illumination

Size of field	Length of side	Area
	cm	cm²
1/5	7 9	62 7
1/4	8 8	78 3
1/3	10 2	104 4
2/5	11 2	125 3
1/2	12 5	156 6
3/5	13 7	188 0
2/3	14 5	208 9
3/4	15 3	234 2
4/5	15 8	250 6
1/1	17 7	313 3

quencies between 2 and 20 per second can be obtained. We adjusted the flicker frequencies in such a way that the faster of the two fields gave flicker frequencies between 10 and 20 a second, by which we obtained the sharpest responses of our animals. Since it was shown for other Arthropods that the critical frequency for flicker fusion is a function of illumination and that the highest frequency of flicker which can be reacted to at all lies between 53 and 62 per second, we are certain that with the intensities and frequencies used we were far below the fusion point

For test young *Limulus* were used varying in length from 4 to 8 cm. Young animals freshly collected are in most cases positively phototropic. They are used for test as soon as they have been brought to the Laboratory. If there are too many animals available at one moment they are kept in a tank with running sea water of low temperature, half shaded in the supply house. If left in the

dark room over a longer period, all animals become negatively phototropic just as older ones do   A change in the sign of phototropism can also occur as a result of handling the animals (Cole (1922-23))   With a sufficient number available it is possible, however, to have always as many positively phototropic animals as needed

One at a time the animals are brought into the tank in dim red light, and they are held in a position on the middle line between the two flickering fields   To keep the *Limulus* in a position to start its run on a course on the bisecting line between the two fields, a slight pressure is exerted on the tip of its tail by means of a glass rod which has a piece of rubber tubing on its end   The animal begins leg movements as soon as the two fields are illuminated and the sector discs are set into motion   It is released at a moment when its body axis is exactly on the bisecting line   It now takes a course toward the two fields, and at about the middle of the tank turns to the right or to the left field toward which it proceeds with considerable speed   Each arrival at one or at the other field is counted   Each animal will give six to ten successive runs which are definite   Pretty soon, however, the animal will during its course turn far to the right or to the left or run away from the lights just as if its phototropism was changed from positive to negative   This could easily be due to the repeated handling during which the animal was brought back to the starting point   As soon as there is any uncertainty about the conduct of an animal, it is eliminated   Before setting the sector discs into motion, the animal is tested several times and its reaction is observed   If there is any tendency for response toward the field of one side which could be due to bodily asymmetry, the animal is at once excluded from tests with flickering fields   For each ratio of flicker frequencies and areas, about 60 to 70 tests are made   It was found that this number of runs is sufficient for quantitative treatment of the data   During each set of experiments carried out with a given flicker frequency and area ratio, the sector discs and diaphragms securing the respective areas were shifted from right to left and from left to right repeatedly so as to avoid any possible influence of the position of a field relative to the animals tested

In the first set of experiments the ratio of areas was kept 1 1 throughout (313 3 cm ^{2} 313 3 cm ^{2}), and the ratio of flicker frequen-

TABLE II

With equal areas equally illuminated having different frequencies of flicker below that critical for fusion (F_1 and F_2) in the ratios given (Γ_1/Γ_2), the number of times is given that animals went to F_1 and to F_2 and proceeded to the midregion (M) between the two flickering fields   The ratios of the numbers for F_1 and F_2 are given as N_1/N_2

F_1/F_2	Field	No of animals							N_1/N_2
		1	2	3	4	5	6	Total	
0 20	1	1	2	0	2	1	1	7	
	5	7	7	12	13	5	3	47	0 149
	M	3	2	0	0	1	0	6	
0 25	1	1	3	2	2	3	—	11	
	4	8	2	12	11	11	—	44	0 250
	M	1	1	2	1	0	—	5	
0 33	1	0	2	2	5	3	1	13	
	3	3	5	8	7	10	5	38	0 368
	M	0	2	0	3	3	1	9	
0 40	2	2	3	1	4	3	2	15	
	5	4	10	5	10	7	3	39	0 385
	M	1	0	1	3	1	1	7	
0 50	1	2	6	4	3	1	—	17	
	2	4	13	6	6	7	—	36	0 472
	M	0	3	2	1	1	—	7	
0 60	3	3	8	2	4	5	—	22	
	5	7	10	4	7	8	—	36	0 611
	M	0	0	2	1	4	—	7	
0 66	2	5	6	4	5	—	—	20	
	3	9	6	6	8	—	—	29	0 690
	M	6	5	2	4	—	—	17	
0 75	3	5	4	5	6	1	—	21	
	4	6	6	7	8	2	—	29	0 759
	M	1	5	5	4	3	—	18	
0 80	4	5	5	5	3	3	—	21	
	5	7	5	4	4	5	—	25	0 840
	M	7	2	3	3	7	—	22	

cies was varied   It soon appeared that the number of animals going to the faster flickering field is always greater than to the slower field In fact the ratios of the numbers of animals counted at each field are proportional to the ratios of the flicker frequencies (Table II)
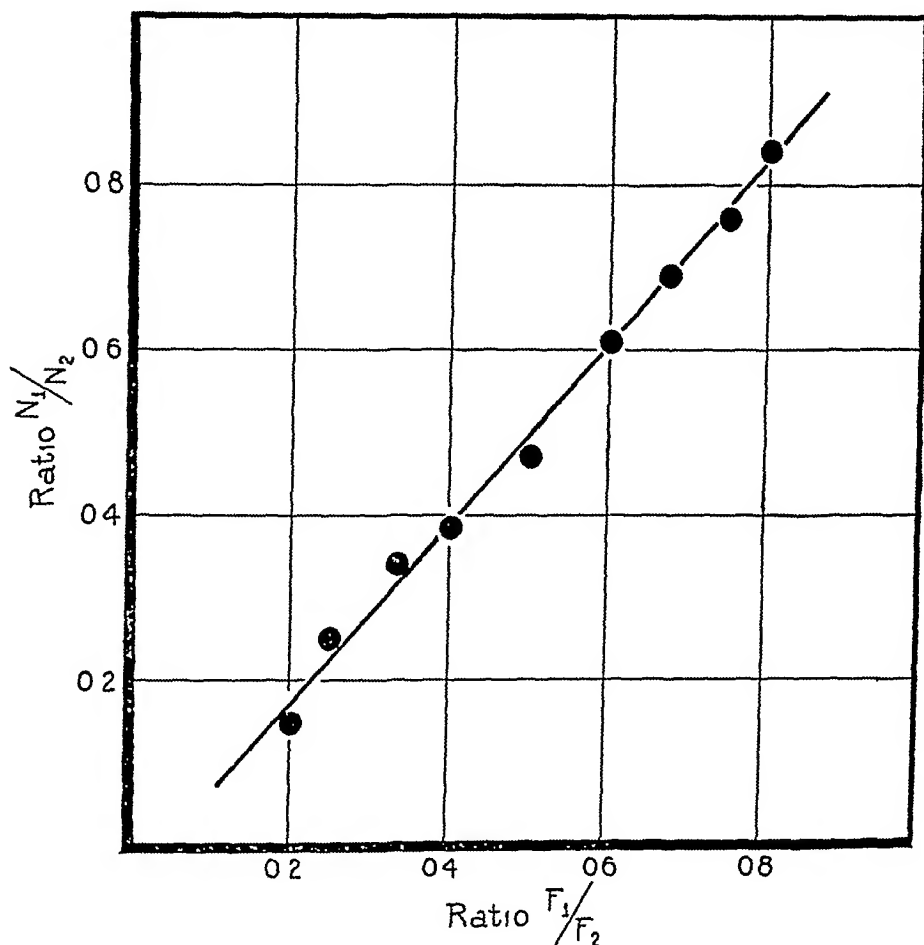
FIG 2   Relations between flicker frequencies of two illuminated fields of equal areas and the number of animals reacting to the fields   The slope of the line is slightly above one

If we plot the ratios of numbers of animals at each field, N_1/N_2, against the ratio of flicker frequencies, F_1/F_2, we obtain a rectilinear relationship (Fig 2)   The same has been found earlier in the honey bee (Wolf (1933), (Wolf and Zerrahn-Wolf (1934–35))   It is interesting, however, to note that the slope of the line in Fig 2 is slightly

above 1   This indicates that the faster flickering field always has a slightly greater effect than the slower field

Since it has been found that between two flickering fields of equal brightness and area but differing in flicker frequency the stimulating effect of the fields is proportional to the flicker frequencies, the question arises whether two fields differing in flicker frequency can be equalized in their stimulating effect by reducing the area of the faster flickering field   From experiments with the honey bee we know that such an equalization can be arrived at   It might be possible also to obtain it by reducing the brightness of the faster flickering field Experiments with the bee showed, however, that the range of variation in this respect is very great before any effect can be noticed

For tests with *Limulus* the area of one field is kept constant (17 7 cm x 17 7 cm), and a sector disc is inserted to provide a given flicker frequency   The area of the other field is made smaller, and its flicker frequency is adjusted so that the ratio of the two areas is inversely proportional to the respective flicker frequencies   If for *Limulus* the same is true as for the bee, we expect a ratio of 1 in the number of animals seeking either field   The results of our tests are given in Table III and in Fig 3   By studying Fig 3 we notice that the mean ratio, for which theoretically we would expect 1, is slightly above 1   The calculated mean = 1 028   If we indicate $3 \times P E_1$ in the graph, all the points are within the limits of this band   The difference between the theoretical mean and the one experimentally found seems just barely significant since $P E_M$ (mean ratio) = 0 0086 It seems therefore that in this series of tests, just as in the previous ones, we have a slightly greater stimulating effect of the faster flickering field

With the new results for the eyes of *Limulus* another piece of evidence for the strong reaction of an Arthropod to flicker below fusion is given   Mast and Dolley (1924, 1925) pointed out that for the photic response of *Eristalis* the effect of intermittent stimulation of low frequency (between 10 and 25 per sec.) is considerably greater than with higher flicker frequencies or with continuous illumination Quantitative evidence for reaction to flicker of low frequency was given in the honey bee (Wolf (1933), Wolf and Zerrahn Wolf (1934–35))   In all the Arthropods tested the relations of area, intensity of

TABLE III

The ratio N_1/N_2 of animals going respectively to illuminated fields 1 and 2, flickered at frequencies F_1 and F_2, is shown for different ratios of the areas of the fields (M records, as in Table II, the number of journeys to the region between the two fields)

Ratio of areas	Ratio F_1/F_2	Field	No of animals							N_1/N_2
			1		3	4	5	6	Total	
1 5	5 1	1	5	5	4	7	6	—	27	0 964
		5	6	5	6	6	5	—	28	
		M	1	2	4	3	1	—	11	
1 4	4 1	1	5	4	4	5	7	4	29	1 036
		4	5	3	6	5	5	4	28	
		M	2	5	2	2	0	2	13	
1 3	3 1	1	4	6	3	5	6	5	29	1 074
		3	6	4	1	6	5	5	27	
		M	2	2	4	1	3	0	12	
2 5	5 2	2	6	3	6	8	3	6	32	1 032
		5	6	4	5	5	6	5	31	
		M	0	0	0	4	3	0	7	
1 2	2 1	1	8	3	7	5	5	6	34	1 030
		2	8	4	8	5	3	5	33	
		M	2	0	0	2	2	1	7	
3 5	5 3	3	6	4	5	8	6	—	29	1 074
		5	9	4	4	6	4	—	27	
		M	1	0	3	2	2	—	8	
2 3	3 2	2	6	3	4	5	2	7	27	0 964
		3	4	5	5	6	2	6	28	
		M	2	2	3	1	2	1	11	
3 4	4 3	3	6	5	4	5	4	—	24	1 043
		4	5	6	3	6	3	—	23	
		M	1	3	5	3	4	—	16	
4 5	5 4	4	6	4	7	5	6	—	28	1 037
		5	3	4	8	7	5	—	27	
		M	3	4	1	2	5	—	15	

light, and flicker frequency lead to the conclusion that by the visual
fields certain numbers of retinal elements are stimulated which give
rise to impulses to the central nervous system and thus cause definite
action of the motor mechanism   In case of areas differing in size and
illumination, it was found that the product of area and intensity has
to be constant to give equal stimulating effects   With varying areas
and flicker frequencies, equivalent stimulating effects are obtained if
for two fields the areas are inversely proportional to their flicker
frequencies   It therefore seems that for equality of stimulation it is

| Ratio of Areas | 15 | 14 | 13 | 25 | 12 | 35 | 23 | 34 | 45 |
| Ratio of FF | 51 | 41 | 31 | 52 | 21 | 53 | 32 | 43 | 54 |
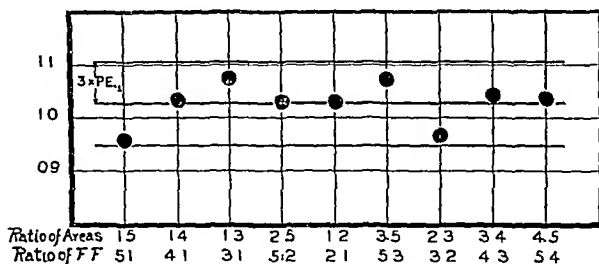
FIG 3 Ratios of number of animals turning to one of two illuminated fields
differing in area and in flicker frequency   The theoretical mean for equal effect
should be 1   It was found to be 1 028, suggesting a slightly superior effect of
flicker as such

irrelevant whether a greater number of elements is stimulated by a
large area which is weakly illuminated or has a low flicker frequency,
or a smaller number of elements is stimulated by a smaller area of
stronger illumination or high flicker frequency   For the coordination
of motion during a tropistic response it seems that the same effect is
obtained when few impulses due to low illumination or low flicker
frequency are coming from a greater number of receptor elements or
when more frequent impulses due to stronger illumination or faster
flicker are sent out from a smaller number of receptor elements

SUMMARY

In phototropic tests with young *Limulus*, the phototropic reactions to flickering fields were studied   If the two fields are equal in area and brightness but different in flicker frequency, the number of animals going to the two fields is proportional to their flicker frequencies   Equal stimulating effects of two fields differing in flicker frequency are obtained by reduction of the area of the faster flickering field   The areas for equal effect must be inversely proportional to their flicker frequencies   It seems that equal effects are dependent upon equality of the number of active excitation elements per unit of time

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CARBOXYPEPTIDASE

II. The Partial Purification of Pro Carboxypeptidase

By M. L. ANSON

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N J)

Extracts of autolyzed pancreas contain carboxypeptidase which even in the presence of formaldehyde can digest chloracetyl tyrosine and peptic digest of edestin. Part I[1] described the isolation of this carboxypeptidase in crystalline form. If fresh pancreas is extracted with cold salt solution, the extract does not attack a formalized peptic digest of edestin. On standing at 37°C., however, the extract slowly becomes active. The activation is enormously hastened by the addition of trypsin. Thus, fresh pancreas contains not active carboxypeptidase (CP) but an inactive precursor, pro carboxypeptidase (PCP). The nature of this precursor is not known. It may be a protein which is different from carboxypeptidase. It may be carboxy peptidase combined with an inhibitor.

Pro carboxypeptidase can be partially purified by fractionation with ammonium sulfate. Most of the pro-carboxypeptidase in the extract is precipitated by 0 35 saturated ammonium sulfate but not by 0 2 saturated ammonium sulfate. The protein can be freed of ammonium sulfate by precipitation by ferric chloride or by dialysis under carefully controlled conditions.

The pro carboxypeptidase in the partially purified preparation, like the pro carboxypeptidase in the crude pancreatic extract, is ac tivated by trypsin. Partially purified pro carboxypeptidase contains trypsinogen, which trypsin can convert into trypsin. The activation of impure pro carboxypeptidase by trypsin, therefore, is partially due to the added trypsin and partially due to trypsin formed from the trypsinogen present. Until pro carboxypeptidase is prepared free from trypsinogen, experiments on the kinetics of activation of pro carboxypeptidase by trypsin are of dubious significance.

Enterokinase can activate pro-carboxypeptidase   This activation may be due to the trypsin formed from trypsinogen by enterokinase In the presence of sufficient trypsin inhibitor no activation takes place   From this result alone one cannot decide whether the inhibitor acts by eliminating activation by trypsin or by interfering with direct activation by enterokinase   The inhibitor does not affect the activity of activated carboxypeptidase

Pro-carboxypeptidase is not activated by a small amount of chymotrypsin   If a large amount of chymo-trypsin is used there is in time a partial activation which may be due to a slight impurity of trypsin

In general the results agree with but do not prove the hypothesis that pro-carboxypeptidase, like chymo-trypsinogen (Kunitz and Northrop (1934–35)) is activated only by trypsin   More conclusive experiments are not possible with the impure pro-carboxypeptidase now available

To estimate pro-carboxypeptidase, trypsin is added and the resulting carboxypeptidase is estimated   Part III[2] describes the activation and estimation of pro-carboxypeptidase and defines the pro-carboxypeptidase unit [PCP u]

Partial Purification of Pro-Carboxypeptidase —Bovine pancreas is trimmed of fat, and rapidly frozen immediately after removal from the animal, and stored frozen   If it is impossible to freeze the pancreas immediately it should at least be promptly chilled in ice water   To each kilogram of ground frozen pancreas are added 3 liters of 5 per cent sodium chloride solution and 150 ml of toluol (Eastman practical)   The suspension is stirred and allowed to stand overnight at 5°C In the morning the toluol and fat are skimmed off the top and the suspension is filtered through very fine gauze   114 gm of ammonium sulfate are added to each liter of solution which makes the solution 0 2 saturated with ammonium sulfate   40 gm of Filter-Cel and 20 gm of Standard Super-Cel (Johns-Manville) are added to each liter of solution and the solution is filtered on a large Buchner funnel through a filter bed of Standard Super-Cel   To each liter of filtrate are added 89 4 gm ammonium sulfate, which makes the solution 0 35 saturated with ammonium sulfate   The suspension is filtered on large folded papers in the cold, the precipitate is hardened on a Buchner funnel, and stored frozen   Half saturated ammonium sulfate precipitates only 10 per cent more pro-carboxypeptidase than 0 35 saturated ammonium sulfate

The extraction of fresh pancreas is less efficient than that of autolyzed pan-

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[1] Page 781

creas In a typical case, the sodium chloride extract contained 0 012 [PCP u]$_{ml}^{PDE}$ and 3 5 mg nitrogen per ml

The specific activity of crystalline carboxypeptidase is 0 01 [CP u]$_{mg}^{PDE}$ $_N$ The specific activity of the ammonium sulfate precipitate after activation is 10 per cent that of crystalline carboxypeptidase and 2 5 times that of the sodium chloride extract Before activation the carboxypeptidase activity of the ammonium sulfate precipitate is less than 3 per cent of its activity after activation

The procedure outlined can be applied to unfrozen fresh pancreas The specific activity of the product obtained is a quarter to a third less than that of the product obtained from frozen pancreas

TABLE I

Activation Experiments

Activator	Time activation	Time digestion	Increase in for mol titration
	min	*min.*	*ml 0 02 N sodium hydroxide*
Trypsin	5	10	0 6
Chymo-trypsin	5	30	0 0
	60	30	0 3
Enterokinase	5	10	0 45
Enterokinase plus inhibitor inhibitor added before enterokinase	5	20	0 05
Enterokinase, inhibitor added after activation but before estimation	5	10	0 40

Pro-carboxypeptidase solution, final concentration in all cases 1 gm un hardened ammonium sulfate precipitate per 100 ml 5 per cent sodium chloride

Trypsin 0 5 × 10^{-4} hemoglobin units (Anson and Mirsky (1933–34)) crystal line trypsin (Kunitz and Northrop (1935–36)) per ml

Chymo-trypsin 0 3 × 10^{-4} hemoglobin units crystalline chymo trypsin (Kunitz and Northrop (1935–36)) per ml

Enterokinase 1 drop of concentrated solution (prepared by Dr M Kunitz) per 2 ml

Inhibitor 5 drops of concentrated solution of protein free crude trypsin in hibitor (prepared from pancreas by Dr Kunitz) per 2 ml

Activation carried out for various times at 37 C 0 5 ml used for digestion of peptic digest of edestin (see Part III) for various times

It is not possible to purify or crystallize pro-carboxypeptidase by the technique used to isolate carboxypeptidase. The ammonium sulfate precipitate is com pletely soluble in barium hydroxide

Precipitation with Ferric Chloride —Pro-carboxypeptidase is very easily de natured by acid One can, however, precipitate 80 per cent of the partially

purified pro-carboxypeptidase with ferric chloride (which is acid) without any change in specific activity   To each gram of ammonium sulfate filter cake are added 10 ml of a cold solution of 5 per cent sodium chloride containing enough ferric chloride to make the solution green to brom cresol green   The precipitate is filtered off in the cold   It is completely soluble at pH 8 0

Dialysis —1 gm of ammonium sulfate filter cake is dissolved by the addition of 16 ml of 0 1 M acid potassium phosphate and 4 ml of 0 1 M di-potassium phosphate   The solution is dialyzed overnight at 10°C in a shaking dialyzer (Kunitz and Simms (1927–28)) against a solution containing 2 per cent sodium chloride, 0 008 M acid potassium phosphate, and 0 002 M di-potassium phosphate   Under these conditions pro-carboxypeptidase can be dialyzed without destruction or activation

Activation Experiments —The experiments and their results are given in Table I

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CARBOXYPEPTIDASE

III. The Estimation of Carboxypeptidase and Pro Carboxypeptidase

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This paper describes the estimation of carboxypeptidase, CP, by formol titration, with chloracetyl tyrosine or a peptic digest of edestin as substrate To estimate the inactive precursor of carboxypeptidase, pro carboxypeptidase [PCP], the precursor is activated with trypsin and the resulting carboxypeptidase is estimated

Peptic digests have not hitherto been used as substrates of car boxypeptidase because they are digested not only by carboxypeptidase but also by other proteolytic enzymes of the pancreas I have found, however, that carboxypeptidase activity is not stopped by formalde-hyde and that the digestion of a peptic digest by a crude pancreatic extract is due entirely to the carboxypeptidase in the extract, if the digestion is carried out in the presence of formaldehyde If a crude extract of pancreas and a given solution of crystalline carboxypeptidase have the same activity as measured with chloracetyl tyrosine, they likewise have the same activity as measured with a formolized peptic digest of edestin

The peptic digest is much cheaper than chloracetyl tyrosine, which is important when many thousands of analyses have to be made Furthermore, the peptic digest is stable at the pH used for estimation, whereas a fresh chloracetyl tyrosine substrate solution has to be made every day

Preparation of Chloracetyl Tyrosine Substrate—To 2 gm of chloracetyl tyrosine (Hoffman La Roche) are added 50 ml of 0 02 M di potassium phosphate, 15 ml of 36–38 per cent formaldehyde solution, 2 ml of 0 5 per cent phenol phthalein in 50 per cent alcohol, enough 0 5 N sodium hydroxide to dissolve the

chloracetyl-tyrosine and make the solution just detectably pink, and finally enough water to make the total volume 100 ml

Preparation of Formalized Peptic Digest of Edestin —To 100 gm of edestin (Pfanstiehl) are added 200 ml 1 N hydrochloric acid, 500 ml of water, 4 gm pepsin (Parke, Davis), and a little toluol   The solution is kept at 37°C overnight and is then filtered   To each 100 ml of filtrate are added 3 gm of decolorizing charcoal (Norit) and 1 gm of the filter aid Standard Super-Cel (Johns-Manville), and the suspension is filtered on a Buchner funnel through a bed of Standard Super-Cel   5 N sodium hydroxide is added to the filtrate until the solution is alkaline to thymolphthalein and the resulting precipitate is filtered off with the aid of Standard Super-Cel   The further addition of sodium hydroxide should cause no further precipitation   It might be thought that the three steps which have just been described could better be combined but the charcoal removes more color if the residue not dissolved by pepsin is first removed and if the solution is kept acid   To each 50 ml of the clear alkaline solution are added 15 ml of formaldehyde, 2 ml of 0 5 per cent phenolphthalein, enough 5 0 N sodium hydroxide to make the solution just detectably pink, and enough water to make the total volume 100 ml   The solution is stored at room temperature and is not used until it has stood overnight   If the solution ceases to be pink more sodium hydroxide is added

Preparation of Standards —The standard pink solutions are kept in tightly stoppered, lipless 50 ml centrifuge tubes   They contain roughly 0 0007 per cent phenolphthalein completely converted into the colored form by an excess of sodium hydroxide   The total volume is roughly 7 5 ml , which is about the volume of the titration mixture   The edestin standard contains 2 5 ml of the peptic digest which has been made alkaline and filtered but to which the water, formaldehyde, and indicator have not yet been added   Thus, both the pink standard and the digestion mixture contain the same amount of brown peptic digest in the same volume and a good match is obtained at the end point of the titration   When the colorless chloracetyl-tyrosine is used as a substrate, the standard is simply an alkaline solution of phenolphthalein

Technique of Formol Titration —In the estimation of proteolytic enzymes by formol titration the usual procedure is to add enzyme to a substrate solution, to stop the digestion after a given time by the addition of formaldehyde, and finally to add phenolphthalein and find out how much sodium hydroxide is needed to make the solution as pink as a standard pink solution   A blank titration is done on a solution to which formaldehyde has been added before the addition of enzyme, *i e* , on a solution in which no digestion has taken place   The increase in titration due to digestion is a measure of the number of amide linkages split   This usual procedure has to be slightly modified for the estimation of carboxypeptidase because carboxypeptidase activity, unlike tryptic activity, is not stopped by formaldehyde   A slightly alkaline substrate solution is used which already contains formaldehyde and phenolphthalein   The blank is obtained by titrating immediately after the addition of enzyme   The digestion is then measured by

titrating 10 minutes after the addition of enzyme   Since the formaldehyde and the indicator are not added separately and since the acid groups "liberated" from the peptic digest are already neutralized in the substrate solution, the modified procedure for formol titration is even simpler than the ordinary procedure

The Blank Titration —1 ml of enzyme solution is added to 5 ml of substrate solution in a tube like that in which the standard is kept, the tube is whirled to mix the solutions, and the mixture immediately titrated with 0 02 N sodium hydroxide until its color matches that of the standard   It is important that the titration be carried out rapidly because digestion goes on and carbon dioxide is absorbed during the titration

The Digestion Titration —If the blank titration with enzyme added is more than 0 2 ml more than the blank titration with water added, then, in order that the digestion mixture should have the proper pH, an amount of 0 02 N sodium hydroxide is added to the substrate solution sufficient to make the blank with enzyme plus sodium hydroxide the same as that with water alone without enzyme or added sodium hydroxide   After the enzyme is added the tube is stoppered and titrated after 10 minutes   The substrate solution is brought to 25°C before the enzyme is added and the digestion mixture is kept in a 25°C water bath during the digestion

If the increase in formol titration is more than 1 8 ml with chloracetyl tyrosine as substrate or 0 8 ml with the peptic digest as substrate then the digestion is repeated with less enzyme or with a shorter digestion time   If the increase in titration with chloracetyl tyrosine as substrate is more than 1 2 ml but less than 1 8 ml then after 6 minutes of digestion 0 7 ml of sodium hydroxide is added to the digestion mixture and the titration is completed after 10 minutes as usual

Estimation of Pro-Carboxypeptidase —The pro-carboxypeptidase is activated with crystalline trypsin (Kunitz and Northrop (1935–36)) and the resulting carboxypeptidase is estimated as already described   The concentration of pro-carboxypeptidase to be estimated should be such that 1 ml of the activated solution can be used directly for the estimation of carboxypeptidase

The trypsin is dissolved in 0 02 N hydrochloric acid to give a solution having 2×10^{-3} hemoglobin units (Anson and Mirsky (1933–34)) per ml   1 drop of this solution is used for each milliliter of pro carboxypeptidase solution   First the trypsin is put in a test tube, then an equal volume of 1 M di potassium phosphate, then the pro-carboxypeptidase   Usually this solution is red to phenol red if it is not, 0 1 N sodium hydroxide is added until the solution is red to phenol red and the volume of sodium hydroxide added is recorded   The test tube is left for 5 minutes in a water bath kept at 37°C

The final solution is slightly alkaline because trypsin acts most rapidly in slightly alkaline solution   The alkali is added after the trypsin and before the enzyme in order to protect the enzyme from the acid in the trypsin solution

Doubling the amount of trypsin or the activation time does not affect the results

The Activity Units —A solution of carboxypeptidase by definition has 1 carboxypeptidase unit per ml ($[CP\,u]_{ml}$) when it causes an increase in formol titration under standard conditions at the initial rate of 1 milliequivalent of sodium hydroxide per minute (*cf* Northrop (1932–33)) An amount of crystalline carboxypeptidase which has 1 mg nitrogen has 0 081 $[CP\,u]^{CT}$ when the substrate is chloracetyl-tyrosine and 0 103 $[CP\,u]^{PDE}$ when the substrate is a peptic digest of edestin Thus 1 $[CP\,u]^{CT}$ is equivalent to 1 27 $[CP\,u]^{PDE}$ Initially the peptic digest is digested by a given amount of carboxypeptidase 1 27 times more rapidly than is chloracetyl-tyrosine

A solution of pro-carboxypeptidase has 1 pro-carboxypeptidase unit $[PCP\,u]$ if after activation with trypsin it has 1 carboxypeptidase unit

Activity Curve —Fig 1 shows the extent to which chloracetyl-tyrosine and a peptic digest of edestin are digested by increasing amounts of crystalline carboxypeptidase in 10 minutes under the standard conditions described The digestion of chloracetyl-tyrosine is proportional to the amount of enzyme until the increase in formol titration is 1 6 ml of 0 02 N sodium hydroxide In the case of the digestion of a peptic digest of edestin the deviation from proportionality begins with very small amounts of digestion and the peptic digests of other easily available proteins are even less satisfactory than the peptic digest of edestin If the substrate concentration is increased then the extent of digestion is proportional to the amount of enzyme for greater extents of digestion This advantage, however, is more than outweighed by the disadvantage that with higher concentrations of substrate the substrate solutions are more highly buffered and hence the titration is less sensitive With the concentrations of substrate used the titrations are sensitive to 0 05 ml of 0 02 N sodium hydroxide

Calculations —If the digestion is carried out with 1 ml of enzyme solution, then the carboxypeptidase units of the carboxypeptidase in 1 ml of solution are read directly from Fig 1 If the digestion time is 5 minutes instead of 10 minutes, then the carboxypeptidase units values must be doubled If the digestion time is 20 minutes, then the carboxypeptidase units values must be halved

Specificity of Formalized Peptic Digest as Substrate —The evidence that in the presence of formaldehyde carboxypeptidase alone is re-

sponsible for the digestion of a peptic digest of edestin by a crude
extract of pancreas is that the same values for the carboxypeptidase
activity of a crude extract are obtained whether chloracetyl tyrosine
or the peptic digest is used as a substrate   It may be that there are
proteolytic enzymes other than carboxypeptidase which attack peptic
digests in the presence of formaldehyde   If there are, their quanti
tative importance in crude pancreatic extracts is small compared with
that of carboxypeptidase   None of the known proteolytic enzymes of
the pancreas other than carboxypeptidase has been shown to be active
in the presence of formaldehyde
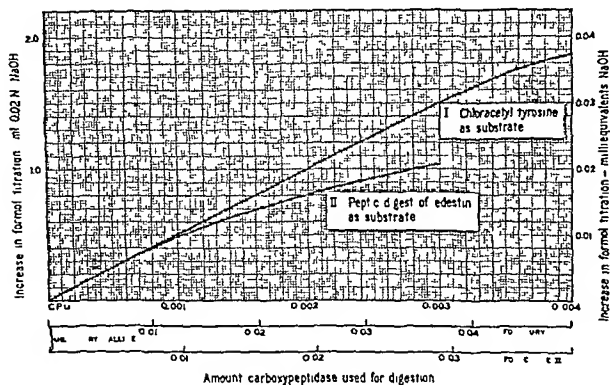
FIG 1

Reproducibility of Formalized Peptic Digest as Substrate —Trypsin
digests different proteins, even samples of edestin prepared in different
ways, at quite different rates   In order to obtain reliable absolute
values for tryptic activity it is necessary to use a highly reproducible
substrate such as hemoglobin   Carboxypeptidase in contrast attacks
the peptic digests of widely different proteins at about the same initial
rate, and in the case of edestin small differences in the preparation of
the peptic digest do not cause any change in the rate at which the
digest is attacked by carboxypeptidase   Since, however, the re
producibility of peptic digests is always suspect, every batch is checked

by means of an enzyme solution whose activity has been measured with chloracetyl-tyrosine as a substrate

Stability of Substrates —The chloracetyl-tyrosine solution changes slowly   It should be kept cold and used the day it is prepared   The formalized peptic digest of edestin does not change in a month when kept at room temperature

Effect of Tyrosine and Glycerine —The digestion of chloracetyl-tyrosine or a peptic digest of edestin by crystalline carboxypeptidase is not changed by the addition to the digestion mixture of 1 ml of 4 per cent glycerine or 1 ml of 0 02 M tyrosine

Effect of Acid —Since acid slows digestion by carboxypeptidase any acid introduced with the enzyme solution is neutralized before digestion is begun   The amounts of acid produced during the digestion of the peptic digest which causes an increase in formol titration of less than 0 8 ml of 0 02 N sodium hydroxide do not affect the rate of digestion   In the digestion of chloracetyl-tyrosine the acid formed is important if it causes an increase in formol titration of more than 1 2 ml of sodium hydroxide   If the final increase in formol titration is to be more than 1 2 ml of sodium hydroxide part of the acid formed by the digestion should therefore be neutralized with sodium hydroxide during the digestion, as has been described

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THE APPARENT DISTORTION OF BRIEF RECTANGULAR ELECTRICAL STIMULI IN NERVE

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It has been shown previously (Blair (1932 a, b, c), (1935 a, b), (1936 a)) that the strength-duration curves of medullated nerve and of numerous other tissues are represented quite well by the equation,

$$\log \frac{V}{V - R} = kt + \log \frac{K + k\alpha}{K} \tag{1}$$

in which V is the stimulating voltage, R the rheobase, t the duration of the stimulus, and k, K, and α are constants. The kinetics assumed for the excitatory state, p, in order to give this result were,

$$\frac{dp}{dt} = KV - kp \tag{2}$$

with the additional assumption that ordinarily the excitatory state, p, need not be built up to a constant threshold value, h, but only to a value $h - \alpha V$, α being a constant. In a few cases a threshold, $h + \alpha V$, was required. It appears that this state, αV, which has been assumed to be a lowering of the threshold but may equally well be any kind of excitatory condition, is ordinarily attained by kinetics which are so fast that most strength-duration data do not enable them to be evalu ated. Some recent data on muscle, however, extend to times which are relatively short enough so that the kinetics of the fast process may be investigated. In this case, the assumption is justified fairly well (Blair (1936 b), data by Colle (1933)) that the fast process may be written,

$$\frac{dh}{dt} = AV - ah \tag{3}$$

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in which h is the alteration of the threshold, V is the stimulus, and A and a are constants   For these data are represented by the equation,

$$p + h = h_0 = \frac{KV}{k}(1 - e^{-kt}) + \frac{AV}{a}(1 - e^{-at}) \tag{4}$$

h_0 being the real threshold of the excitatory state   Or, on putting in the condition for the rheobase, R,

$$\frac{V - R}{V} \times \frac{K + ka}{K} = e^{-kt} + \frac{ka}{K}e^{-at} \tag{5}$$

It appears from this point of view that the constant, a, is so large in stimulating most nerve trunks, that e^{-at} is negligible, even with the briefest stimuli, so that the second member of equation (4) appears only at the equilibrium value, AV/a, or aV Data by Sakamoto (1933) from the stimulation of single nerve fibers with micro-electrodes give, however, values of a which are sufficiently small to enable the fast component of equation (4) to be followed   Four sets of these data are given in Table I, with both the observed voltages and those calculated from equation (4), using the constants given

The method used in applying equation (5) is illustrated in Fig 1 Since this equation, for large values of t, reduces to equation (1), plotting log $V/(V - R)$ against t for the longer times gives k from the slope of the line obtained and $(K + ka)/K$ from the intercept   In Fig 1, the straight line from the upper part of the curve is continued as a broken line over to this intercept in each case   Having determined these constants, it will be seen that a is the only undetermined constant remaining   Its mean value may be obtained by substituting each datum at short durations and averaging the results

It will be observed in Table I that with only two data do the observed and calculated voltages diverge by as much as 5 per cent This indicates that equation (4) is valid to within the limit of error usually allowed in this type of measurement

The durations corresponding to the least voltages in Table I are the measured utilization times of the rheobases   These data enable a determination of the completeness of the components represented by $\frac{KV}{k}(1 - e^{-kt})$ at these times   In Experiment 10, for example,

$1 - e^{-kt} = 0\,94$, approximately, and in Experiment 2, $0\,996$, approximately These are the extreme cases, the least and the most complete, respectively Since the other component of equation (4) is entirely complete at these times and since it is an appreciable part of
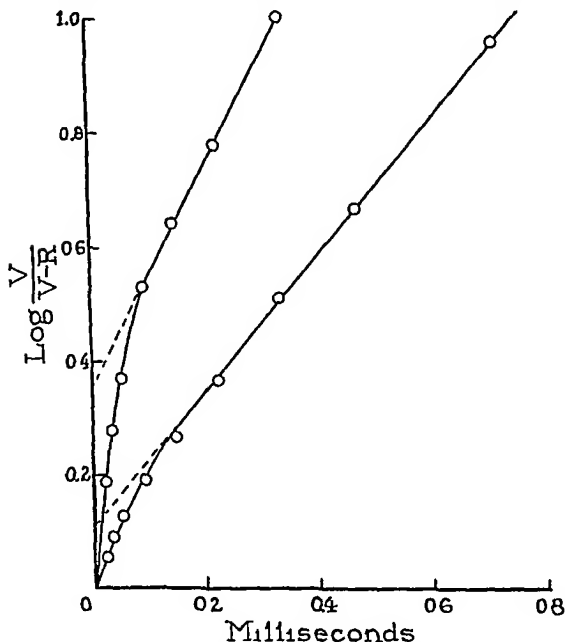
FIG 1 The data of Experiments 3 and 9 (Table I) upper and lower curves, respectively, plotted according to equation (1)

h_0, it will be evident that even in Experiment 10, the apparent incompleteness corresponds to only about 4 per cent of the value of R Therefore, any neglected factor, such as accommodation, cannot be considered to have an appreciable effect in these cases

It will be seen from Table I that $a = 8k$, approximately   The component in $(1 - e^{-at})$ of equation (4) will therefore be sensibly complete in one-eighth of the utilization time of the rheobase, $i e$, in about 0 15 millisecond   At shorter durations, the incompleteness of this component causes the points in Fig 1 to diverge from the linear

TABLE I

Examples of Sakamoto's Data Using His Numbering for the Experiments

The calculated voltages are obtained from equation (4) using the given constants, k, etc

t	Exp 9		Exp 10		Exp 2		Exp 3	
	V		V		V		V	
	Observed	Calculated	Observed	Calculated	Observed	Calculated	Observed	Calculated
msec								
0 0251	30 1	28 4					23 8	23 4
0 0255			33 5	33 2	29 4	28 6		
0 0377	20 9	20 2	23 4	23 9	21 6	22 2	17 6	18 1
0 0566	15 3	15 0	18 2	18 3	18 6	17 8	14 5	14 7
0 0943	10 9	10 8	13 5	13 7	14 6	14 4	11 9	12 2
0 151	8 49	8 25	11 0	11 1	12 2	12 3	10 9	10 8
0 226	6 75	6 75	9 42	9 42	11 0	11 1	9 9	9 9
0 339	5 64	5 65	8 22	8 00	9 95	10 0	9 26	9 25
0 471	4 96	4 98	6 94	7 09	9 78	9 48	8 88	8 85
0 716	4 37	4 37	6 21	6 25	9 39	9 05	8 93	8 50
1 03							8 34	8 35
1 04	4 00	4 08	5 44	5 76	8 97	8 95	8 34	8 34
1 13					8 90	8 90		
1 17			5 42	5 42				
1 26	3 88	3 88						
	k = 2700		k = 2400		k = 4800		k = 4400	
	a = 20,000		a = 24,200		a = 33,000		a = 32,000	
	$\dfrac{K + k\alpha}{K} = 1\ 28$		$\dfrac{K + k\alpha}{K} = 1\ 38$		$\dfrac{K + k\alpha}{K} = 1\ 76$		$\dfrac{K + k\alpha}{K} = 2\ 26$	

relations   Existing data from exciting nerve trunks do not show this type of divergence, indicating that the factor, a, in the trunk is much larger so that the component $(1 - e^{-at})$ attains its limiting value, 1, in close approximation even with the briefest stimuli   The probable values of a in these cases are discussed later

With regard to the meaning of equation (4), it may be supposed that instead of the states, p and h, there is only the one, p, given by,

$$p = \frac{KV}{k}(1 - e^{-kt}) + \frac{AV}{a}(1 - e^{-at}) \tag{6}$$

or, on differentiating,

$$\frac{dp}{dt} = V(K + Ae^{-at}) + \frac{AkV}{a}(1 - e^{-at}) - kp \tag{7}$$

If now it is assumed that the process of excitation proper is given simply by,

$$\frac{dp}{dt} = K'I - kp \tag{8}$$

the first terms of equation (7) will represent the distortion of the current in passing through the tissue, in other words, the application of a constant voltage, V, will give rise to a current of the form,

$$I = V(K + Ae^{-at}) + \frac{AkV}{a}(1 - e^{-at}) \tag{9}$$

From this it will be seen that I decreases according to

$$\frac{dI}{dt} = a(I_0 - I) \tag{10}$$

I_0 being the final value of I on long application of V. Therefore, instead of assuming as before (Blair (1936 b)) a fast excitatory process in addition to the ordinary excitatory process, it can equally well be assumed that there is only the one process but that the exciting current undergoes a rapid exponential diminution from its initial to its final value

This transient in the current is probably due to the electrical capacity of some structure which is not the site of excitation. Its time constant may appear, therefore, in purely physical measurements of the electrical properties of the tissue

Physical measurements on excitable tissue have not as yet been related satisfactorily to excitation phenomena. It is well known,

however, that there are transients in nerve trunks   These have been recorded directly, *e g*, by Bishop (1928)   Other measurements with alternating currents (*e g* Lullies (1930)) show also that the nerve trunk is a complex structure electrically   It is, of course, not necessary that there should be any simple correspondence between the total current through the tissue and the actual exciting current, but there may be simple relations between the time constants of the transients of the one and the other   Bishop (1928) concluded that the transient he measured was complete at about the same time as that at which the excitatory state became adequate   Consequently, he supposed that the polarization of the tissue and the state of excitation might be simply related   This transient had a time constant about the value, k, of equation (6)   One difficulty with this interpretation, however, is that k is quite definitely not a property of the trunk but of the fiber, since the k of each fiber may be separately determined from the intact trunk (Blair and Erlanger (1933), Blair (1934)) without changing the electrodes   The constant, a, which probably is a property of the trunk when the whole trunk is being stimulated, may appear in alternating current measurements   These measurements have been interpreted by Cole (1932) as indicating that the nerve trunk has a variable impedance element of constant phase angle   It appears, however (Cole and Curtis (1936)), that an alternative interpretation is that each fiber has only simple capacities and resistances but that the combination of fibers in the trunk may appear to have properties corresponding to the earlier interpretation

In any case, it is concluded by Cole from these measurements (Blair (1936 *b*), discussion) that there is a factor in the frog's nerve trunk analogous to a time constant of the order of 10^6 sec $^{-1}$ According to the data considered here, $a = 30,000$ sec $^{-1}$, approximately, for single sciatic fibers, but the strength-duration curves for sciatic trunks require values at least as great as 10^5 sec $^{-1}$, because in them the term e^{-at} of equation (6) is negligible at times as short as about 3×10^{-5} sec   It appears, therefore, that if the transients are due to capacities, in stimulating the trunk, the capacities of the single fibers are encountered in a series arrangement since the effective capacity of the whole is less than that of the elements   There appears to be some indication, therefore, that both the physical measurements and the physiological

measurements will give a similar result with respect to this fast transient

It will be seen that equation (7) is unnecessarily complex in order to conform to previous notations (Blair (1936 b))   It may be written,

$$\frac{dp}{dt} = V(M + Ne^{-t}) - kp \tag{11}$$

M and N being constants   When the stimulus is stopped, the transient will probably be reversed so that the excitatory state will disappear according to,

$$\frac{dp}{dt} = - NVe^{-at} - kp \tag{12}$$

$i\,e$, the transient will be contra excitatory at the cathode, and the excitatory state will disappear faster than its spontaneous rate, kp

According to the data considered here, the sign of A/a in equation (4) is positive always, indicating a rapid decrease in the exciting current, due perhaps to a series capacitance   There are cases, particularly in the α excitability of muscle (Blair (1936 b)) however, in which the sign of this constant is negative, corresponding to a transient increase in the exciting current, possibly due to a parallel capacitance with respect to the exciting circuit proper   Considerations of this kind may be of value in determining the locus of the excitatory state

It cannot be concluded without considerable investigation that the current transient is accurately exponential   This is in fact rather unlikely, but it may be that it is sufficiently nearly exponential that no great divergence will be detected by strength duration data   Also it seems likely, according to Bishop's observations (1928) that the excitatory state itself has an external electrical sign   Consequently, the excitatory state and the transient in the current will probably appear in a combination of some sort in purely physical measurements

This transient, or rather the last term in equation (1) has been discussed by Rushton (1934) and Hill (1936) from separate points of view

Hill assumes that it does not exist and asserts that the first two terms of equation (1) are entirely sufficient in representing strength

duration curves   There are some few data for which this is true, *i e*, the transient is unimportant   Most data, and those considered here are examples, do not conform, however, to Hill's assumption even in rough approximation   With their own data, using alternating currents as stimuli, Hill, Katz, and Solandt (1936) conclude in agreement with the writer (1932 *b*) that the formula corresponding to equation (1) is not valid for high frequencies   It cannot be said without further investigation that the neglect of the transient is the only reason for this, but its inclusion accounts qualitatively for the experimental results

Rushton (1934) did not question the existence of the third term of equation (1) but criticized it, first on the ground that it had been assigned no physical meaning, and second on the ground that the state represented by it (called change of threshold then) would disappear instantaneously on the removal of the stimulus so that it should not effect the data at all in certain specified cases

Regarding the first point, Rushton may be satisfied by considering the effect concerned to be a current transient

Regarding the second point, Rushton's assumption, which, as he showed, leads to absurdities, is entirely his own   For it is quite clear Blair (1932 *b*))[1] that no such idea was entertained by the writer, although he was unable then to state explicitly an exponential decay as is done now by the first two terms of equation (12)

SUMMARY

If it is assumed that the kinetics of the process of excitation in nerve is given by $dp/dt = KI - kp$, I being the actual exciting component of the current, p the state of excitation, and K and k constants, it is necessary to postulate that on application of a rectangular stimulus of voltage, V, the current, I, undergoes a transient exponential variation, usually a decrease, in order that the integral of the differential equation (above) may fit the strength-duration data in V and t This hypothesis is substantiated by data by Sakamoto on single fibers of the sciatic nerve of the frog   The time constant of the postulated current transient is of the order of 10^{-4} sec for single fibers and of the order of 10^{-5} sec or less in the sciatic nerve trunk   The latter value is

[1] Page 723, third sentence

about the same as that found by Cole in the same tissue by purely physical measurements Some criticisms by Rushton (1934) are discussed

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IMMUNOLOGICAL STUDIES ON PEPSIN AND PEPSINOGEN

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The experiments to be described were carried out in the attempt to
distinguish by serological methods the pepsins from several different
animal species, as well as to compare the serological behavior of pepsin
and its precursor, pepsinogen

The concept of organ specificity was established by Uhlenhuth (1) who showed
that the lens protein of one species would give rise to antibodies having no rela
tion to the serum proteins of the same species, although these antibodies would
react with lens proteins from unrelated species   Enzyme proteins have been
shown to be antigenic. Sumner and Kirk (2, 3) were able to produce a pre
cipitating serum with crystalline urease, and to demonstrate the protective effect
of such antibodies against the toxicity of the enzyme *in vivo*   Although the anti
urease would completely remove the urease from solution, the resulting precipi
tate apparently retained most of its activity   Northrop (4) found that crystal
line swine pepsin protein gave rise to pepsin precipitating antibodies   By means
of the Dale technique TenBroeck (5) was able to differentiate pig and beef trypsin,
as well as chymotrypsin and its precursor, chymotrypsinogen, each of which
had been purified by five crystallizations

Pepsin presents a special problem since, according to Northrop (4),
it is inactivated above pH 6, as a more alkaline condition is approached
the enzyme is converted into a typical denatured protein   At 37°C
and pH 7 6, almost all of the pepsin is inactivated or denatured imme
diately, and on being returned to pH 2–3, the denatured pepsin precipi
tates   It is therefore most likely that active pepsin cannot exist in
the body fluids, and that inactive, denatured pepsin is responsible for
antibodies developed following the injection of active pepsin   North
rop (4) and Kirk and Sumner (3) have commented on this likelihood,
and it must be borne in mind in the interpretation of these experi
ments, since the denaturation of a protein may produce a new speci
ficity very different from that of the original native protein (8–11)

Attempts to carry out precipitin reactions in the acidity range at which pepsin is native revealed the fact that between pH 4 and 6 both pepsin and pepsinogen precipitate normal serum proteins   For example, if a normal rabbit serum is adjusted to pH 5, and pepsin at pH 5 is layered over it, there will be a definite ring formation, even at a pepsin dilution of 1 1,000,000   This is also true of normal chicken, bovine, swine, and horse sera, and of the purified albumin and globulin fractions of normal horse serum   At the normal serum pH of 7 6 no precipitation occurs

The coagulation of milk by pepsin, one of the most delicate tests for peptic activity, is carried out at pH 6   Using this test to demonstrate antipeptic activity of pepsin precipitating sera, it was observed that several normal rabbit sera showed a greater inhibition of activity than did the pepsin antisera   It is possible that this nonspecific inhibition is related to the nonspecific precipitation, since both take place in about the same range of acidity   Apparently it is impossible to study specific serological reactions with active pepsin as such, the limitations imposed by its denaturation at pH 7 6 must be accepted

EXPERIMENTAL

Rabbits weighing about 2 kg were given three intraperitoneal injections at weekly intervals of 5 0 cc of a 1 per cent solution of swine pepsin   This material had been twice crystallized, and dialyzed   It was injected at pH 5   2 weeks after the last injection, the rabbits were bled and serum collected   Pepsin was prepared for the precipitin reaction by adjusting a concentrated solution to pH 7 6, and diluting to 1 per cent on the basis of dry weight of pepsin   This solution, referred to as 1×10^{-2} was diluted 1 10 serially to 1×10^{-6} Precipitin reactions were done by the ring test, layering antigen dilutions over the undiluted antiserum, and reading after $1\frac{1}{2}$ hours at room temperature   Of four rabbit sera prepared as described above, two showed no pepsin precipitins, one precipitated pepsin at a concentration of 1×10^{-3}, and one at 1×10^{-5} Although the two positive sera gave similar results, the experiments presented will deal only with the stronger serum   Antisera precipitating swine serum proteins were prepared by injecting rabbits intramuscularly with swine serum adsorbed onto alumina according to the method of Heltoen (6)   The strongest serum so obtained, which precipitated a 1×10^{-5} concentration of swine serum protein (on the basis of dry weight), was used

Pepsinogen (7) gives rise to precipitating antibodies more readily than does pepsin   At pH 7 6 it is a stable native protein   Four rabbits were given three intraperitoneal injections at weekly intervals, of 5 0 cc of a 1 per cent solution

of pepsinogen at pH 7 6 2 weeks after the last injection, two sera showed a precipitate at 1 × 10⁻⁵ (1 gm enzyme protein in 100 000 cc salt solution) concentration of pepsinogen two sera reacted at 1 × 10⁻⁶ 3 weeks after the last injection the titers were the same at which time the animals were exsanguinated, and serum collected The pepsinogen preparation used in these rabbits was later shown to contain a certain amount of swine protein other than pepsinogen

Pepsin

Through the kindness of Dr Northrop samples of cattle, rabbit, guinea pig, chicken, and shark pepsin were available These were tested for precipitation at pH 7 6 in swine pepsin antiserum The results appear in Table I Only the swine and bovine pepsins were crystallized, the amount of pepsin in the other preparations was estimated by activity measurements and expressed in Table I as rennet units The amount of enzyme in 1 0 cc which will clot 10 cc of 20 per cent "Klim" (pH 6) in 1 minute is defined as 1 rennet unit In the case of the crystallized swine pepsin, 1 rennet unit per cubic centimeter represents a concentration of about 2 × 10⁻⁵ gm per cubic centimeter on the basis of dry weight of pepsin protein

It is apparent that swine and bovine pepsin react equally well, guinea pig considerably less so, and the other pepsins not at all in the swine pepsin antiserum The same swine pepsin antiserum was absorbed with these heterologous pepsins, and tested thereafter with swine pepsin

In the homologous absorption using swine pepsin, a solution containing 100 rennet units per cubic centimeter was added to an equal amount of undiluted antiserum, incubated overnight at 37 C , and the precipitate removed in the centrifuge. The supernatant fluid no longer precipitated swine pepsin in the range of dilutions tested If a smaller absorbing dose was used, 30 to 10 rennet units per cubic centimeter, the titre of the serum was reduced about 100 times

Table II shows the extent to which swine pepsin precipitins were removed by heterologous pepsins In this and the following tables, each symbol represents one tube, the first, an antigen dilution of 1 × 10⁻² (1 gm enzyme protein in 100 cc salt solution), the second, 1 × 10⁻³, and so on, the highest dilution being 1 × 10⁻⁶ Tubes showing a definite ring after 1 1/2 hours at room temperature are designated +, those with a faint ring ± Thus, + + + − − indicates a titer of 1 × 10⁻⁴

It appears that bovine and guinea pig pepsins completely remove the swine pepsin precipitins Rabbit, chicken, and shark pepsins do not affect these antibodies, nor does swine serum In addition to native swine serum proteins, HCl (pH 2) denatured serum proteins were also used and found to be unreactive in swine pepsin antisera

TABLE I

Reaction of Heterologous Pepsins in Swine Pepsin Antiserum

Pepsin tested*	Rennet units *per cc*						
	100	30	10	3	1	0 3	0 1
Swine pepsin	+	+	+	+	+	±	—
Bovine pepsin	+	+	+	+	+	+	±
Guinea pig pepsin	+	+	±	—	—	—	—
Rabbit pepsin		—	—	—	—	—	—
Chicken pepsin	—	—	—	—	—	—	—
Shark pepsin		—	—	—	—	—	—

* All negative in normal rabbit serum

TABLE II

Absorption of Swine Pepsin Antiserum with Heterologous Pepsins

Absorbed with	Rennet units *per cc*	Subsequent titer with swine pepsin
Saline*		++++−
Swine pepsin	100	−−−−−
Bovine pepsin	100	−−−−−
Guinea pig pepsin	100	−−−−−
Chicken pepsin	100	++++−
Shark pepsin	30	++++−
Rabbit pepsin	10	++++−
Swine serum		++++−

* Serum diluted 1 2 with physiological salt solution as a control on the serum dilution effected by absorption

It is true that the rabbit and shark pepsins in the highest concentrations available would be expected only to reduce the titer of the serum, but no evidence of such a reduction could be detected These findings confirm the direct precipitation reactions Bovine and guinea pig pepsins cross-react with swine pepsin precipitins, while rabbit, chicken, and shark pepsins probably do not According to Nuttall (12) there

is some serological relationship between the serum proteins of swine and cattle, and none whatever between swine and the other species tested, including the guinea pig   By means of an antiserum precipitating swine serum proteins, similar results were obtained   Bovine serum precipitated and absorbed swine serum precipitins incompletely, guinea pig and chicken sera not at all   It was also found that swine pepsin was not reactive with the swine serum protein precipitins   The use of a 1 per cent solution of swine pepsin in salt solution as a diluent did not alter the reaction of swine serum protein with its homologous antiserum

Pepsinogen

The undiluted antisera prepared with swine pepsinogen reacted with that material in a concentration of 1×10^{-6} (1 gm pepsinogen protein in 1 million cc of salt solution)   Tested with swine pepsin, there was a faint reaction at 1×10^{-5} and 1×10^{-6}, the lower dilutions being negative   Swine serum proteins also precipitated in pepsinogen antiserum at a dilution of 1×10^{-4} The absorption method was used to determine whether these cross-reactions were due to the known impurity of the injected pepsinogen, or whether the three antigens, pepsin, pepsinogen, and swine serum, were serologically related   The data are presented in Table III   The pepsinogen preparations used here had been more thoroughly purified

It may be seen that the faint reaction of pepsin in undiluted pepsinogen serum described above is eliminated by dilution of the pepsinogen serum 1 2   Moreover, the addition of pepsin to the pepsinogen antiserum left both the pepsinogen and swine serum precipitins unaltered   Complete pepsinogen precipitin absorption did not affect the swine serum protein precipitins   The removal of the swine serum protein precipitins did not affect the pepsinogen titer

The reaction of swine pepsinogen in a swine serum protein precipitating serum was also investigated   It was found that swine pepsinogen reacted with such an antiserum in proportion to its purity, indeed this reaction was useful to a certain extent as a criterion of purity   The cruder preparations often precipitated in a dilution of 1×10^{-4}, as purification proceeded the titer would drop, without loss of enzyme activity, to 1×10^{-2}, an end point corresponding to

1×10^{-5} concentration of swine serum proteins   In some preparations, no precipitation occurred   Several attempts to absorb or inhibit swine serum precipitins with such purified pepsinogen were negative

Reaction of Pepsinogen in Pepsin Antiserum

It has been shown that pepsin reacts only feebly and with a marked prozone in undiluted pepsinogen antiserum, and that dilution of the serum 1 2 is sufficient to eliminate the reaction in the range of dilutions tested   In addition (Table III) pepsin fails to inhibit the reaction of pepsinogen in such a pepsinogen antiserum   However, when pepsinogen was tested in pepsin antiserum, it was found that pure pepsinogen not only precipitated at a concentration of 1×10^{-3}, but

TABLE III

Absorption of Swine Pepsinogen Antiserum

Absorbed with	Subsequently tested with		
	Pepsinogen	Pepsin	Swine serum
Saline*	+++++	-----	+++--
Pepsinogen	-----	-----	+++--
Pepsin	+++++	-----	+++--
Swine serum	+++++	-----	-----

* Serum diluted 1 2 as a control on the serum dilution effected by absorption

also absorbed pepsin precipitins as well as pepsin itself   Absorption of the serum with pepsin eliminated the pepsinogen reacting component as well   These results appear in Table IV   They indicate a true cross-reaction between pepsinogen and antipeptic antibodies   The fact that pepsinogen, however purified, absorbs pepsin precipitins is less significant than the removal of the pepsinogen precipitins by pepsin, for it is difficult to be certain that the pepsinogen preparations are free from small amounts of alkali-denatured pepsin

It has been impossible by chemical means, including three crystallizations, to bring the precipitation end-point of pepsinogen in pepsin antiserum below a concentration of 1×10^{-3} In addition to other fractionation methods, calculated to remove alkali-denatured pepsin, advantage was taken of the fact that the heat denaturation of pepsin-

ogen is reversible by cooling, while that of alkali denatured pepsin is practically irreversible   A solution of purified pepsinogen was heated to 80°C for 5 minutes and cooled at 35°C for 1 minute, followed by the addition of an equal volume of half saturated NaCl solution After removal of the precipitate, the clear supernatant contained 50 per cent of the original pepsinogen   From the properties of the substances involved, complete removal of denatured pepsin might be expected   However, the "reversed" pepsinogen still precipitated at a concentration of 1×10^{-3} in pepsin antiserum

TABLE IV

Absorption of Swine Pepsin Antiserum

Absorbed with	Subsequently tested with	
	Pepsin	Pepsinogen
Saline	++++−	++−−−
Pepsin	−−−−−	−−−−−
Pepsinogen	−−−−−	−−−−−

Pepsin from Purified Pepsinogen

The experiments so far described have dealt with swine pepsin purified by two crystallizations   The serological behavior of a pepsin formed from pure pepsinogen but not subsequently purified was investigated   Table V shows the reactions of a pepsinogen solution before and after activation to pepsin, tested in both pepsin and pepsinogen antisera   Precipitin reactions after two crystallizations are also given

From the fact that twice crystallized pepsin fails to react with a 1 2 dilution of pepsinogen antiserum, one might expect pepsin from purified pepsinogen to behave similarly   However, after the pepsinogen was converted to pepsin, the titer of the resulting solution was only 10 times less than that of the original pepsinogen   Two crystallizations of this pepsin from pepsinogen eliminated the precipitating substance

It was also possible to remove the precipitating substance by the previously described heat denaturation of pepsinogen and subsequent reversal by cooling   Experimental conditions were such that only

10 per cent of the heat-denatured pepsinogen reversed to the native form   Separation of native from the denatured proteins was effected by salting out and filtering off the denatured protein   The soluble native pepsinogen so obtained precipitated at a concentration of 1×10^{-5} in the pepsinogen antiserum, after activation no precipitation could be detected in any concentration

These experiments indicate the presence of an antibody for a material other than pepsinogen in the pepsinogen antisera   Its presence was further confirmed by the fact that absorption of pepsinogen antisera with activated pepsinogen did not affect the pepsinogen precipitins   The nature of this material is not known   It cannot be sero-

TABLE V

Activation of Pepsinogen

Solution tested	Titer in pepsin antiserum 1 2	Titer in pepsinogen antiserum 1 2
Pepsinogen before activation	++---	+++++
Activated pepsinogen (pH 2 18 hrs , readjusted to pH 7 6)	++++-	++++-
After one crystallization	++++-	+----
After two crystallizations	++++-	-----

logically identified with the serum proteins, nor with proteins extracted from the muscle wall of the stomach   Since the fractionation after heat denaturation failed to alter the enzyme activity per milligram of protein nitrogen, it may be concluded that there is probably less than 5 per cent of the material present, the activity and nitrogen estimations are accurate to within about 5 per cent

DISCUSSION

The broad specificity possessed by the swine pepsin antiserum, reacting with the pepsin of an animal species whose serum proteins are unrelated to swine serum proteins, may be due to the necessary use of alkali-denatured pepsin   It has been shown (8–11) that denaturation of a protein brings about a loss of its original specificity   Antisera prepared with such denatured materials have a wider range of reactivity than native protein antisera   Thus denatured horse serum

albumin antiserum reacts with similarly treated albumin from ox and man

The absence of serological cross-reaction between swine pepsinogen and swine serum proteins, both native proteins, is parallel to the absence of cross reaction between the lens and serum proteins of the same species (1)   In the serological relationship between pepsin and pepsinogen, another analogy to the behavior of denatured proteins may be found   Antisera made with native proteins do not generally react with denatured proteins, whereas denatured protein antisera react with both the denatured and native materials   If one substitutes pepsinogen for the native, and pepsin for the denatured protein, the situation is the same   In such experiments it is never certain that all of the protein has been denatured, or if it has, that reversal of denaturation has not occurred   Possibly enough native protein remains to produce native protein antibodies   This does not explain the fact that pepsin, containing less than a millionth part of pepsinogen, will absorb the pepsinogen reacting component from a pepsin antiserum, and leave the same component of a pepsinogen antiserum intact   It seems reasonable to assume that pepsin stimulates the production of a group of antibodies reacting more or less indiscriminately with pepsin and pepsinogen   The antibodies developed to pepsinogen, however, react only with that substance   Of especial interest in this connection are experiments of Michaelis (13) and Landsteiner and van der Scheer (14) who found that proteins treated with pepsin and HCl failed to precipitate in native protein antisera, although they would cause the formation of antibodies reacting with both the pepsinized and native proteins

After conversion of pepsinogen to pepsin, the persistence of a material reacting with pepsinogen antisera has been observed   It probably is due not to incomplete activation of pepsinogen, but to a serologically distinct substance which can be separated from the resulting pepsin by two crystallizations, and from pepsinogen by heat denaturation and reversal   Its nature is not known

SUMMARY

1 Alkali (pH 7 6) denatured pepsins from swine, cattle, and guinea pigs precipitate in swine pepsin antiserum   Similarly treated pepsins from the rabbit, chicken, and shark do not

2 Pepsin antisera react with both pepsin and pepsinogen, but do not react with the serum proteins from the homologous species

3 Pepsinogen antisera react with pepsinogen, but not with twice crystallized pepsin, nor with the serum proteins from the homologous species   Positive reactions between activated pepsinogen and pepsinogen antiserum have been observed   It was possible to remove the reacting material from either the pepsinogen or the activated pepsinogen mixture

4 Antisera made with serum proteins do not react with the homologous pepsin or pepsinogen

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THE INFLUENCE OF LIGHT AND CARBON DIOXIDE ON PHOTOSYNTHESIS*

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I

INTRODUCTION

Abundant evidence has accumulated to show that the primary reactions in the photosynthetic mechanism involve a cyclical process consisting of a photochemical reaction and a temperature sensitive reaction ("dark" or "Blackman reaction") This concept has been based on the studies made by Blackman (1905) on the effect of temperature and light intensity on the rate of photosynthesis and expressed by him as "the law of limiting factors " But it was the studies later made by Warburg (1919) which definitely showed the need for interpreting the properties of the system as a two reaction process Emerson and Arnold (1932 *a, b*) using intermittent illumination have made a thorough study of these cyclical reactions, and have contributed much to our knowledge of them Thanks to the work of these investigators and others, this concept serves as one of the main bases for further progress in the understanding of the photosynthetic mechanism

Starting with the system as a cyclical process, several investigators (Stoll, 1932, 1936, Franck, 1935, Gaffron and Wohl, 1936) have recently considered certain reactions as possibly being involved in photosynthesis These discussions have revolved for the most part about the properties shown by chlorophyll *in vitro* and on quantum yields and the energies involved in possible reactions, and have neglected quantitative treatment of the reaction kinetics On the other hand, many schemes have been proposed for the kinetics of the

process, particularly in relation to light and carbon dioxide (e g , Baly, 1935, Burk and Lineweaver, 1935, Arnold, 1935)

In order to evaluate the many suggestions regarding mechanism and kinetics, it is necessary to have definitive measurements of the kinetic relationships covering a range sufficient to render them critical The existing data do not cover the necessary range or are of inadequate precision Moreover, it has not been demonstrated that measurements made with one plant show fundamentally the same properties as with another We have therefore made extensive measurements with one plant for the effect of CO_2 concentration and light intensity, and have compared them with the previous data for other plants under conditions which show their basic similarities and differences

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II

Apparatus and Procedure

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One of the principal difficulties connected with previous studies on the effect of light intensity has been the inability to achieve a high intensity of illumination without serious temperature disturbance Emerson (1929) *records a maximum* intensity of about 100,000 meter candles, which was just about sufficient to reach the maximum rate of photosynthesis under the conditions of his experiments However, in order to be really certain of the form of the intensity-photosynthesis curves, it is necessary to have measurements which definitely indicate the maximum rate of photosynthesis An arrangement was therefore set up whereby a maximum intensity of 282,000 meter candles (Lux) was achieved It is shown diagrammatically in vertical section in Fig 1

The source was a 500 watt projection lamp A condenser consisting of two plano-convex lenses 4½ inches in diameter and 5½ inches focal length formed an image of the filament approximately in the plane of a projection lens 18 inches from the condenser This lens was also plano-convex of 7½ inches focal length and 4½ inches in diameter, it formed an image of the condenser in the plane of the bottom of the manometer vessel The condenser was suitably diaphragmed in order to reduce the amount of stray light so that the illuminated area at the bottom of the vessel was just sufficient to cover it when the manometer was being shaken The amount of light was approximately doubled by the use of a spherical mirror behind the lamp Since the thermostat was constructed of solid opaque walls, the entire apparatus had to be mounted at an angle in a copper tray and a plane surface mirror mounted in the bath to reflect the light upward Movement of the surface water produced by the shaking of the manometers did not affect the beam of light, since the light entered the water of the thermostat some inches below the surface To prevent deterioration of the mirror mounted in the

water, it was necessary to place it in a brass case protected with aquarium cement on the silvered surface

The intensity of the light was varied with neutral filters made by uniformly exposing 5 by 7 inch photographic plates   Calibrations were made by placing an opal glass plate at the level of the bottom of the manometer vessel and measuring the transmitted light directly with a Macbeth illuminometer   The absolute total brightness was determined in the same way by correcting for the transmission of the opal glass plate   In order to be certain that the filters used were neutral with regard to the visible spectrum, check calibrations were made using a filter (Corning No 246) which transmitted only wave lengths longer than about 580 mμ The values so obtained were identical with the white light values
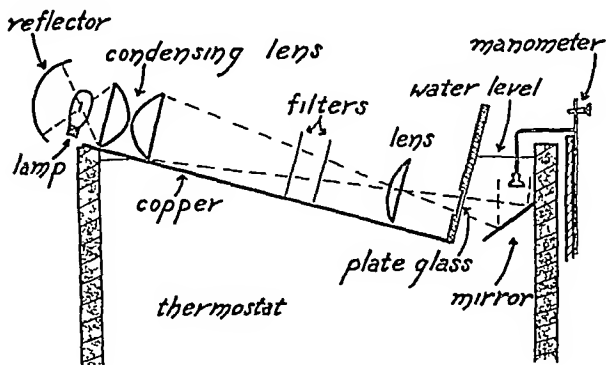
Fig 1 A diagram in vertical section of the apparatus

Photosynthesis was determined as oxygen produced using the Warburg manometric method (Dixon, 1934)   Since this method is now well known, only details of importance in this research are described   One experimental vessel was used with two thermobarometric controls containing the same solution as in the experimental vessel   The volume of the experimental vessel used in all the experiments was 9 858 cc to the level of the Brodie's fluid, and was determined with the mercury method described by Dixon   4 or 5 cc of buffer solution were used and the vessel constants computed were always corrected for the volume of plant tissue in the particular experiment   In all of the experiments described below, the temperature was kept constant at 25 3 C $\pm$ 0 005°

The sources of carbon dioxide were the carbonate bicarbonate mixtures described by Warburg (1919) using the potassium salts as recommended by Emerson and Arnold (1932a)   The carbon dioxide concentrations were recomputed using the

more recent data of MacInnes and Belcher (1933) for the change in the dissociation constants with ionic strength at 25°C   From the law of mass action (Warburg)

$$[CO_2] = \frac{[KHCO_3]^2}{[K_2CO_3]\, K_1'/K_2'}$$ (1)

In logarithmic form, this equation becomes   .

$$\log [CO_2] = 2 \log [KHCO_3] - \log [K_2CO_3] + pK_1' - pK_2'$$ (2)

where $pK_1' = - \log K_1'$, and $pK_2' = - \log K_2'$ According to MacInnes and Belcher, the following empirical expressions hold at 25°C

$$pK_1' = pK_1 - k_1\mu$$ (3)
$$pK_2' = pK_2 - k_2\mu^{\frac{1}{3}}$$

where μ is the ionic strength and the experimentally determined values are pK_1 = 6 343, pK_2 = 10 252, k_1 = 0 119, and k_2 = 0 382   Although their determinations of pK_1' cover a range of ionic strengths below those used here, the change of pK_1' with μ is so small that the extrapolation to higher values is probably justified   Their determinations of pK_2' are within the range of values used here   In Table I are presented the values computed using the above equations and data   Warburg's values are presented for comparison   The CO₂ concentrations computed from the data of MacInnes and Belcher are from 0 042 to 0 073 log units lower than those found by Warburg, which is not a very serious difference considering that all of the data are displaced in the same direction

In order to obtain a solution giving a higher CO₂ concentration than any of these buffer mixtures, tenth molar KHCO₃ was used   Its CO₂ concentration was computed from the following formula which gives a very close approximation (Clark, pp 562–563, 1928)

$$\log [CO_2] = pK_1' + \log [HCO_3^-] - pH$$

$[HCO_3^-]$ was regarded as equal to $[KHCO_3]$, and pK_1' was obtained from the formula of MacInnes and Belcher given above   A glass electrode was used to measure the pH, which is somewhat variable even with the freshly prepared solution always used in these experiments   An average value for $\log [CO_2]$ equal to -3 0 was obtained, a value which is probably not in error by more than a tenth of a log unit   None of the data are seriously affected, since in this solution the rate of photosynthesis is so high that it does not change significantly with the CO₂ concentration

In all of the experiments described, the common aquarium plant *Cabomba caroliniana* was used   Small fronds of about 100 mg wet weight were sufficiently active to give good measurements   It was found that after an equilibration period the same piece of tissue would give constant readings for many hours as

long as the buffer mixtures were renewed often enough to prevent an effective decrease in CO_2 concentration This enabled us to make entire runs with either CO_2 or light intensity as the variable on the same piece of tissue Although smaller pieces of tissue (taken nearer the apex) were more active per milligram (wet weight), identical curves were obtained regardless of the amount of tissue used

Measurements of the rate of respiration made at the beginning of a run were always lower than those made after the plant had been carrying on a high rate of photosynthesis Since a small change in respiration rate has a large effect on measurements made at low photosynthesis rates, the respiration value used in correcting rate of photosynthesis was that obtained at the beginning of a run

TABLE I

Carbon Dioxide Concentrations of Carbonate Bicarbonate Mixtures

No of mixture	Concentration in moles per liter		Ionic strength (μ)	Moles of CO_2 per l ter $\times 10^8$	Log CO_2 concentration	Log CO_2 concentration (Warburg)
	K_2CO_3	$KHCO_3$				
1	0 085	0 015	0 27	0 481	−6 318	−6 276
2	0 080	0 020	0 26	0 902	−6 045	−6 000
3	0 075	0 025	0 25	1 49	−5 826	−5 770
4	0 070	0 030	0 24	2 29	−5 640	−5 585
5	0 060	0 040	0 22	4 48	−5 349	−5 276
6	0 050	0 050	0 20	8 67	−5 062	−5 009
7	0 035	0 065	0 17	20 5	−4 689	−4 638
8	0 025	0 075	0 15	37 5	−4 426	−4 366
9	0 015	0 085	0 13	78 7	−4 104	−4 041
10	0 010	0 090	0 12	131	−3 882	−3 824
11	0 005	0 095	0 11	290	−3 537	−3 481

The correction for respiration does not significantly change the values obtained at high rates of photosynthesis

Plants kept in the dark for some time before the beginning of an experiment gave more reproducible respiration values than plants taken directly from the aquaria where they were kept under a moderate illumination The plant was therefore kept in the dark in buffer for at least 1 hour before beginning an experiment After equilibration for 15 minutes, the respiration was determined for one half hour At low photosynthetic rates, measurements were made for 20 or 30 minutes at high rates, duplicate 5 minute readings were taken Before each new determination 10 to 15 minutes were allowed for the plant to attain the new stationary state During a light intensity run, fresh buffer mixture was used often enough to prevent an effective decrease in CO concentration With carbon dioxide concentration as the variable two readings were made with each buffer,

the plant was then rinsed and placed in a mixture of higher CO_2 concentration Runs were always made starting with the lowest CO_2 concentration or intensity

III

Measurements

1 Light Intensity —In Fig 2 and Table II are presented the data for photosynthesis in relation to intensity obtained on two successive
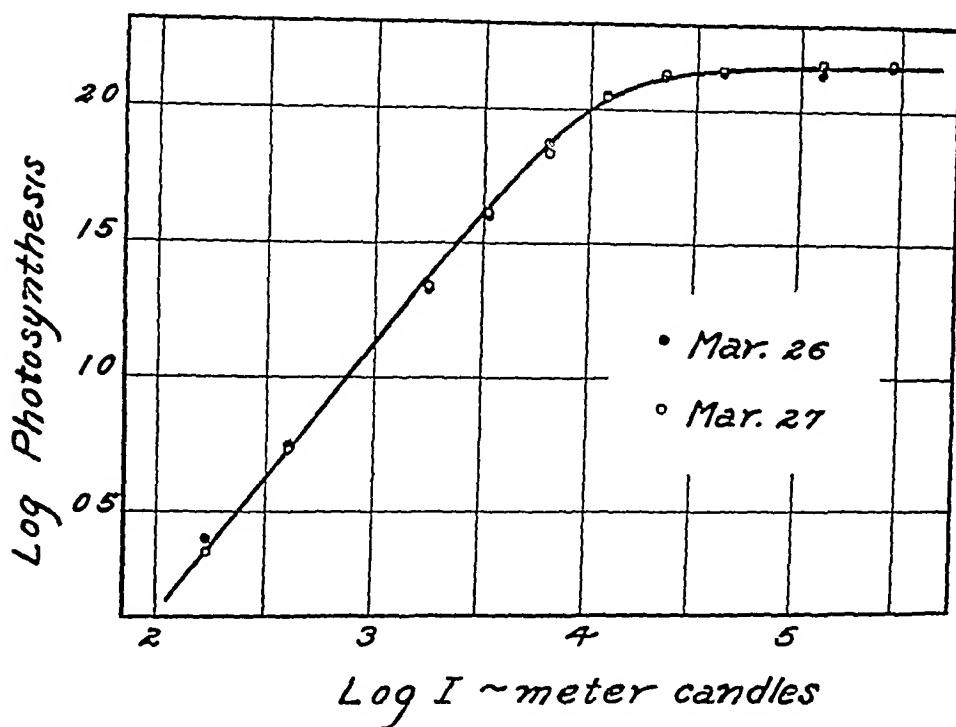
FIG 2 Two runs made on the same frond of *Cabomba* on successive days There is no systematic difference between the two runs   The data are given in Table II   The curve is that of equation (4)

days using the same piece of tissue for both runs   It is clear from these data that individual runs yield data of good precision and that the tissue does not change significantly over a period of 24 hours Similar results have been obtained on many occasions   Although the data of the individual runs are sufficiently critical for the type of equation which represents them, in order to achieve greater certainty

TABLE II

Photosynthesis at Different Intensities   Two Runs on Same Tissue

Data of Fig 2   CO concentration constant at $1\,31 \times 10^{-4}$ moles per liter
Vessel constant $= 0\,535$ Wet weight of tissue $= 116\,5$ mg   Temperature $=$
$25\,3°C$.   Photosynthesis given as c mm of oxygen evolved per hour per 100 mg
wet weight of material corrected for respiration   Respiration measured initially
for 30 minutes

Duration of each reading	Intensity	Rate of photosynthesis	
		March 26 1936	March 27 1936
min	*meter candles*		
20	166	2 52	2 25
20	407	5 56	5 42
10	1 740	21 1	21 7
10	3 310	39 4	40 9
5	6 310	74 0	67 5
5	11,800	112	109
5	21 900	131	135
5	41 700	138	142
5	123 000	139	150
5	282,000	147	149

TABLE III

Photosynthesis and Light Intensity   Detailed Data of Fig 3

Each set of data represents the averages of 5 similar experiments   Photo
synthesis given as c mm of oxygen evolved per hour per 100 mg wet weight of
tissue, corrected for respiration   White light used   Temperature $= 25\,3°C$

Intensity	Rate of photosynthesis			
	[CO$_2$] = 2 05 × 10^{-6} moles per liter	[CO$_2$] = 7,87 × 10^{-6} moles per liter	[CO$_2$] = 1.31 × 10^{-4} moles per liter	[CO$_2$] = 2 90 × 10^{-4} moles per liter
meter candles				
166	1 42	2 99	2 48	2 44
407	4 42	5 41	4 96	5 84
1,740	16 2	27 5	22 2	27 4
3,310	23 2	43 1	42 0	47 4
6 310	31 1	74 3	72 7	91 3
11 800	37 8	104	108	136
21 900	41 3	128	135	164
41 700	41 9	127	145	186
123 000	44 5	140	152	193
282 000	45 2	136	153	192

at low rates of photosynthesis five runs were made with each buffer and the data averaged

In Table III and Fig 3 are given the average data for rate of photosynthesis as a function of intensity for four different carbon dioxide
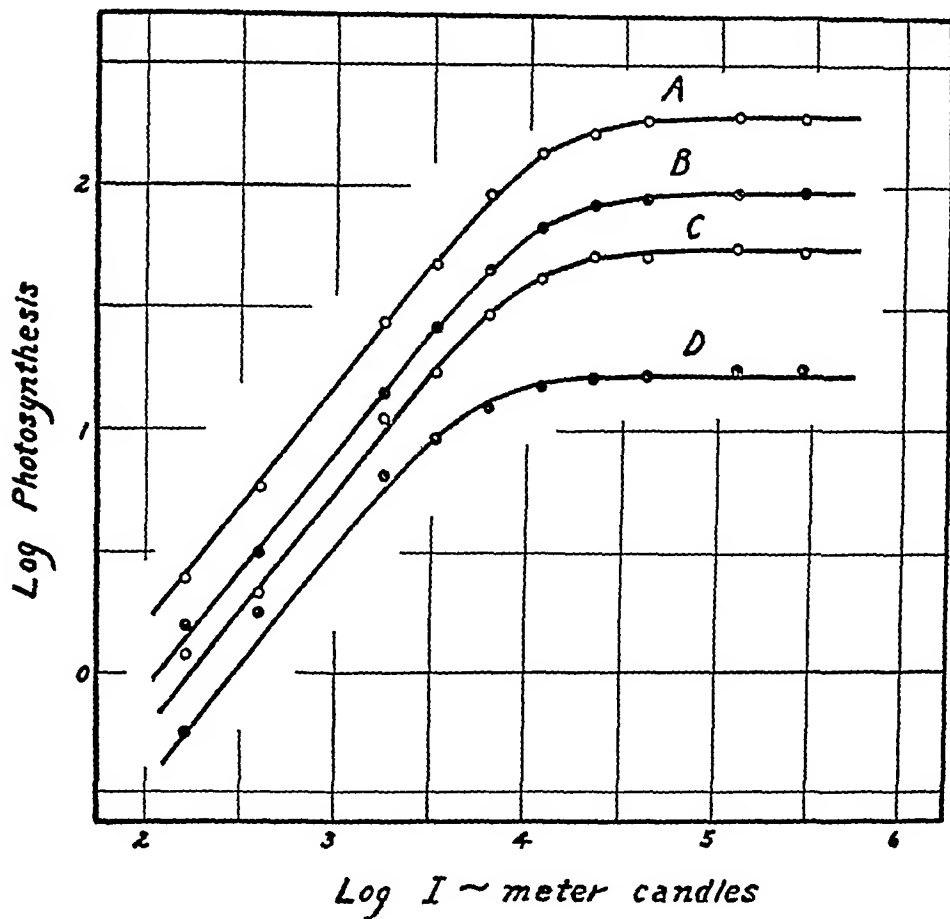
FIG 3 Photosynthesis as a function of light intensity for *Cabomba* The data are given in Table III The photosynthesis scale is correct only for curve *A* The others have been shifted downwards in order to keep the curves distinct *B* by 0 2, *C* by 0 4, and *D* by 0 4 of a log unit The CO₂ concentrations in moles per liter were *A*, 2 90 × 10⁻⁴, *B*, 1 31 × 10⁻⁴, *C*, 7 87 × 10⁻⁵, *D*, 2 05 × 10⁻⁵ The same curve is drawn through all of the data and is from equation (4)

concentrations The data are plotted as log photosynthesis against log *I* with the same curve drawn through all four sets of data It will be observed that the curves for different CO₂ concentrations differ in the intensity at which the maximum rate of photosynthesis is attained,

in accord with Blackman's idea of limiting factors This is also shown by the measurements of Harder (1921), and of Hoover, Johnston, and Brackett (1933)

The curve drawn through the data in Figs 2 and 3 has the equation

$$KI = \frac{p}{(p^2_{max} - p^2)^{\frac{1}{2}}} \tag{4}$$

where p is the rate of photosynthesis at light intensity, I, K is a constant which indicates the position of the curve on the I axis and p_{max} is the asymptotic maximum rate of photosynthesis Equation (4) solved for log p gives

$$\log p = \log p_{max} - 1/2 \log (1 + 1/K^2 I^2) \tag{5}$$

If log p is plotted against log I, the shape of the curve is independent of the constants K and $p_{m\ x}$ This property of the equation facilitates comparison with the data Curves similar to those in Figs 2 and 3, but differing in slope and in inflection, result from changing the exponents in equation (4) An equation which yields a curve very similar to that of equations (4) and (5) may be written as

$$KI = \frac{p}{(p_{max} - p)^{\frac{1}{2}}} \tag{6}$$

Equation (6) solved for log p yields

$$\log p = \log KI + \log [(K^2 I^2 + 4 p_{max})^{\frac{1}{2}} - KI] - \log 2 \tag{7}$$

The curves described by equations (5) and (7) differ slightly only in the rate at which they become parallel to the log I axis at high illuminations The three upper sets of data in Fig 3 fit equation (5) better, while the lowest set of data fit (7) with higher precision Since no certain choice is at present possible and because a majority of the individual data decide for (5) the same curve has been drawn through all four series Exponents other than those in (4) and (6) are definitely excluded, as for example, in the equation

$$KI = \frac{p}{p_{max} - p} \tag{8}$$

or in logarithmic form

$$\log p = \log p_{max} - \log (1 + 1/KI) \tag{9}$$

Equations (5), (7), and (9) have all been drawn to the same maximum in Fig 4 for comparison   It will be observed that all three equations have the same slope at low intensities

It is interesting to note the similarity between the above equations and those derived by Hecht (1923, 1935) for the photosensory process which have been used so successfully to describe many of the proper-

$$1 \quad KI = p/(p_{max}^2 - p^2)^{\frac{1}{2}}$$

$$2 \quad KI = p/(p_{max} - p)^{\frac{1}{2}}$$

$$3 \quad KI = p/(p_{max} - p)$$
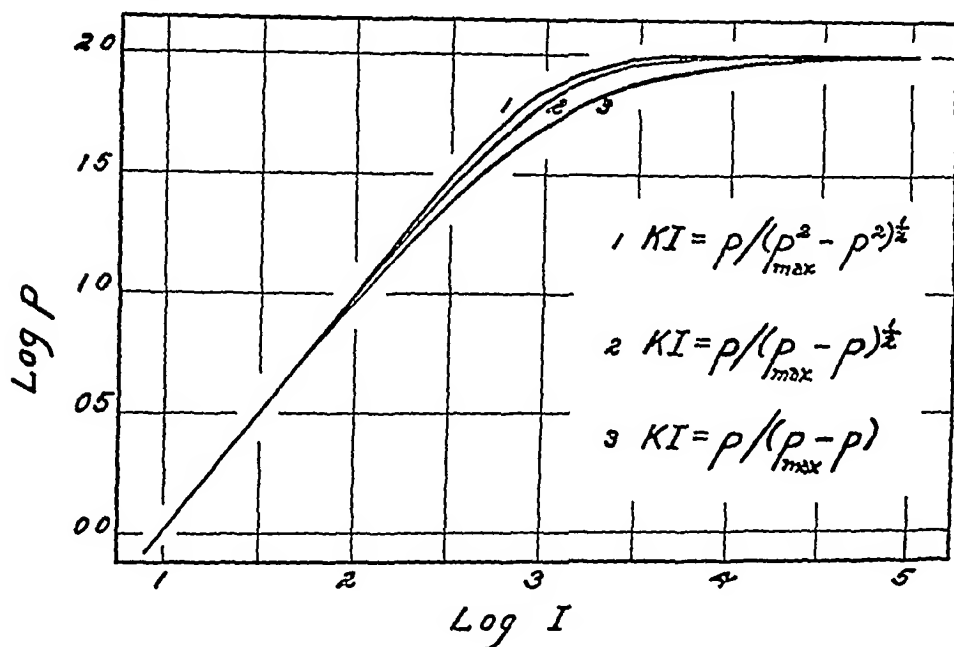
FIG 4   The relation between photosynthesis and intensity in terms of equations (4), (6), and (8)   Plotted on double logarithmic scale, the shape of these curves is independent of the constants in the equation   These equations are similar to those which describe the photostationary state for the photosensory process (Hecht, 1935)

ties of photoreception   In fact, this study began as the result of a comparison between the basic processes of photoreception and photosynthesis   Both are of a cyclical pseudo-reversible character, consisting of a photochemical reaction with a low temperature coefficient and a dark reaction with a high Q_{10} which restores the light absorbing substances to their original condition   The subsequent properties of the reactions are quite different   In one case, nerve endings are stimulated, in the other, carbohydrate is formed

2 *Carbon Dioxide* —Measurements were made of the effect of CO_2 concentration on photosynthetic rate at constant intensity   Since respiration rate was independent of CO concentration, an initial measurement made in the buffer of lowest CO_2 concentration was
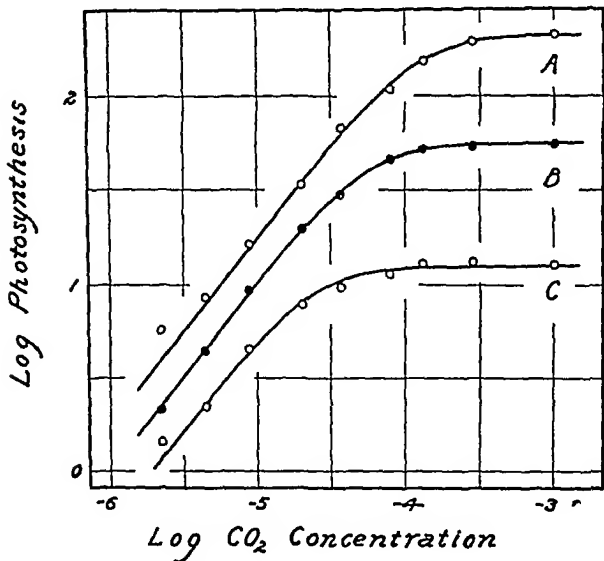
FIG 5 Measurements on *Cabomba* with different carbon dioxide concentrations at constant light intensity   The data are given in Table IV   The scale is correct for curve A, curve B has been moved down 0 4, and curve C, 0 6 of a log unit   No 246 Corning filter was used   The relative intensities were A, 282,000, B, 21 900 and C, 6,310   These are the intensities in meter candles of the un filtered light   The curve drawn through the data is from equation (4)

used in correcting all the photosynthesis rates determined for a given piece of tissue

Because of the time necessary for changing buffers and allowing for equilibration to light and temperature with each new mixture, the

duration of a run was about 5 hours   A continuous exposure to the high light intensities used in these experiments for such a long period occasionally caused a small decrease in rate to take place after 3 or 4 hours   It was found that this decrease could be virtually eliminated by using the long wave lengths of the visible spectrum   Therefore, in all of these experiments Corning filter No 246 was used   This filter is of the sharp cut-off type transmitting 40 per cent of the energy at 588 mμ and 5 per cent at 579 mμ The effective energy was not decreased by more than half, which with the highest intensity avail-

TABLE IV

Photosynthesis and CO₂ Concentration   Data of Fig 5

Each value represents the averages of 5 similar experiments   Red light used, obtained with Corning filter No 246   Intensities are the values in meter candles as determined for the unfiltered light   Photosynthesis as c mm of oxygen evolved per hour per 100 mg wet weight of tissue, corrected for respiration Temperature = 25 3°C

$[CO_2] \times 10^5$ moles per liter	Rate of photosynthesis		
	$I = 6,310$	$I = 21,900$	$I = 282,000$
2 29	5 65	5 33	5 75
4 48	8 79	11 0	8 59
8 67	17 7	23 2	16 4
20 5	31 2	49 2	33 7
37 5	38 0	75 1	68 0
78 7	44 8	115	109
131	50 5	131	152
290	51 9	136	195
1000	50 1	138	212

able did not decrease the rate of photosynthesis measurably   On the other hand, those portions of the spectrum which contribute little energy for photosynthesis but which are injurious to the photosynthetic mechanism were eliminated (*cf* Emerson, 1935)   The use of this red filter changes the intensity values obtained with the white light calibrations   The intensities given are those for white light and may be regarded as only relative values

Fig 5 and Table IV present the rate of photosynthesis as a function of CO₂ concentration for three different illuminations   Each curve

represents the averages of five similar runs   Intensity curves cannot
be derived accurately from these data since the absolute rate of photo-
synthesis varies somewhat with the weight of the tissue as mentioned
above   For example, in the runs with $I = 21,900$, the average weight
of the tissue was considerably lower than in the run with $I = 282,000$
The former therefore gave higher rates per 100 mg than the latter at
low CO_2 concentrations   However, this does not affect the shape of
the curve describing photosynthesis as a function of carbon dioxide
concentration

The curve drawn through the data in Fig 5 is the one used in Figs
2 and 3 and is from equation (4) with carbon dioxide substituted for
light intensity   Apparently the rate of photosynthesis for *Cabomba*
varies in the same way with both light intensity and CO_2 concen-
tration

<div style="text-align:center">IV</div>

<div style="text-align:center">*Data of Other Investigators*</div>

1 Light Intensity —What relation is there between the data pre
sented in this paper and the data obtained by other investigators?
Early experiments over a small range of intensities indicated a linear
relation between photosynthesis and intensity   Reinke (1883)
showed with the bubble counting method on *Elodea* that at high light
intensities a maximum rate of photosynthesis is attained which is not
affected by subsequent increases in the intensity of the light   Aver
ages of his measurements as well as the later ones of Pantanelli (1903)
show good agreement with equation (4) in spite of the crudity of the
method used   The first modern measurements made under satis
factory conditions and with a correction for respiration are those of
Willstatter and Stoll (1918)   Their measurements with several
different species and with both green and yellow leaves also show
excellent agreement with equation (4)   In Fig 6C are drawn two
representative curves from their data   In Fig 6 are also presented
the data of several other observers   None of these is adequately
represented by equation (8), but those of Warburg fit equation (6) a
little better than they do (4)   Other measurements which cover a
smaller range of intensities are those of Van den Honert (1930) made
with *Hormidium* which are omitted as they are identical with the

later ones of Van der Paauw (1932) on the same material   The data of Emerson and Green (1934 *a*) on the marine alga *Gigartina* show good agreement with equation (4)
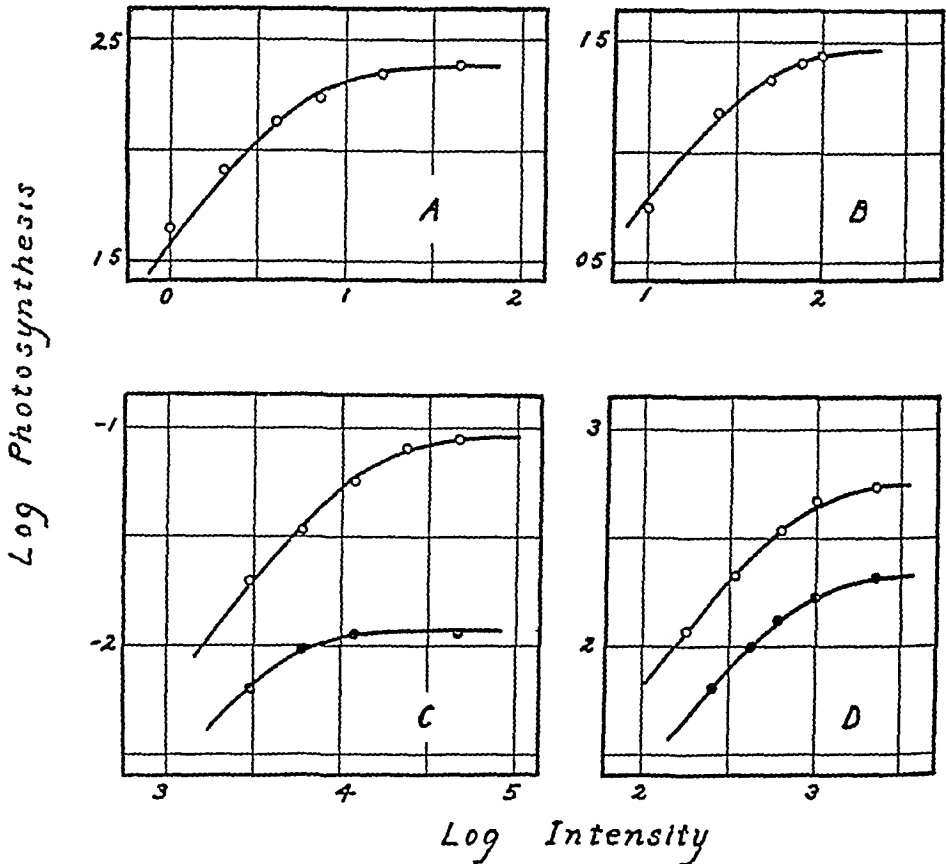
FIG 6 Photosynthesis as a function of light intensity, the data of various investigators   A—Warburg on *Chlorella*, B—Emerson and Green on *Gigartina*, C—Willstatter and Stoll on *Ulmus* yellow leaves (open circles), and on *Ampelopsis* (solid circles), D—Van der Paauw on two varieties of *Hormidium*, Pringsheim's strain (open circles) and Van den Honert's strain (solid circles)   The data are given in the original units of the various authors   The curve drawn through the data is from equation (4)

The data of Emerson (1929) on two strains of *Chlorella* with different amounts of chlorophyll are drawn in Fig 7   It may be noted that on this double logarithmic plot these two curves are evidently of similar shape, whereas on the basis of a semilogarithmic plot Emerson stated

that these curves "are quite dissimilar, and the upper one cannot be produced by multiplying the bottom one by a constant " These data are adequately represented only by equation (4) with K having approximately the same value for both chlorophyll concentrations The point so obviously off the lower curve is a measurement in the region where photosynthesis is smaller than respiration, the pressure
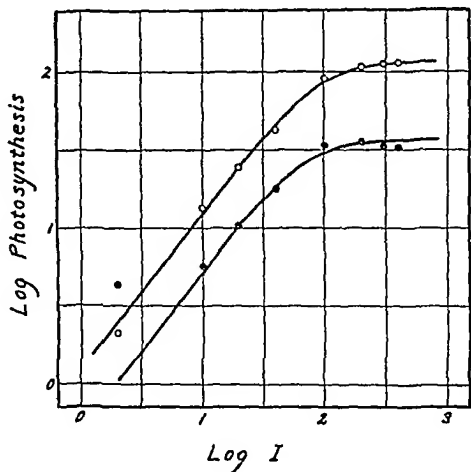
FIG 7 Emerson s data on two strains of *Chlorella* one of high (open circles) and the other (solid circles) of low chlorophyll concentration The same curve has been drawn through both sets of data and is from equation (4)

change is very small and the measurements are therefore of low precision

The intensity measurements of Hoover, Johnston, and Brackett (1933) on young wheat covering a small range of low intensities at various carbon dioxide concentrations are consistent with all the other data discussed above   The data of Harder (1921) on *Fontinalis* as well as numerous other observations in the literature mainly made

from an ecological point of view have too high an experimental error to be critical

Considering the variety of plants, of experimental conditions, and of method, it is remarkable that all of these data give such a good fit with respect to an equation as specific in form as the one drawn through them

2 *Carbon Dioxide* —Comparison of previous results with ours is difficult because the method of supplying CO_2 influences the results Warburg supplied CO_2 from buffer mixtures similar to those used here,
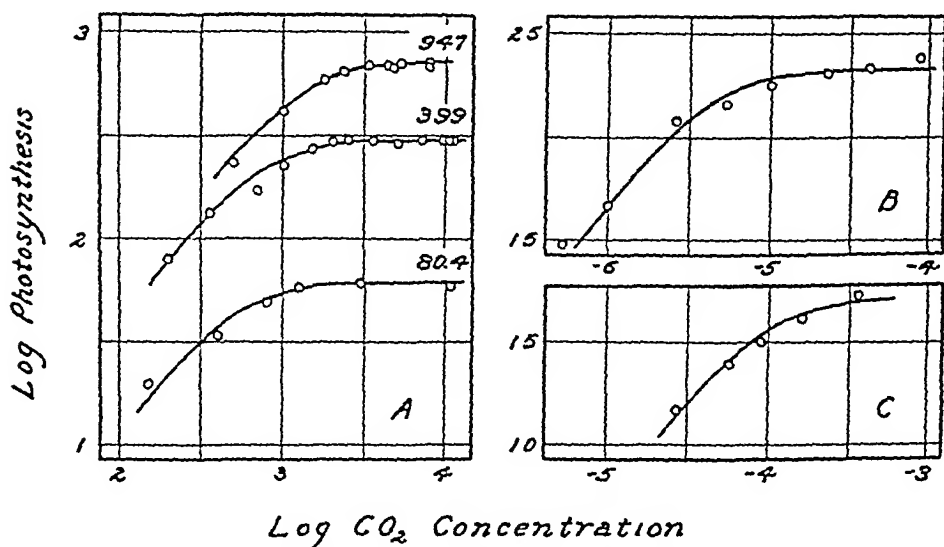
Fig 8 *A* The data of Hoover, Johnston, and Brackett on young wheat The numbers on the curves give the light intensity in foot candles *B* Warburg's data on *Chlorella* *C* Those of Emerson and Green on *Gigartina* The same curve as in the preceding figure has been drawn through these data

his data can therefore be compared directly with ours   This is done in Fig 8*B*   The agreement with equation (4) is not so good as desired, this may be because the data represent only single experiments   The data fit equation (6) better but do not exclude (8)   The work of Emerson and Green on *Gigartina* (Fig 8*C*) is complicated by the use of buffers with a high salt content and a different piece of tissue for each determination   The small range of concentrations makes impossible a choice between the various equations although the data

are not inconsistent with equation (4)   The data of Harder on *Fonti nalis* are omitted as we cannot be certain that a constant CO_2 supply is provided at low CO_2 concentrations by proportionate dilutions of a bicarbonate solution   Moreover, there are too few points available for testing these data

Fig 8*A* gives some of the data obtained with young wheat by Hoover, Johnston, and Brackett   These three curves as well as their others give a good fit with equation (4) and cannot be adequately described by equation (6) or (8)   In these experiments, CO_2 was supplied in gas mixtures circulated rapidly through an enclosed chamber   However, the data of Van den Honert and Van der Paauw on *Hormidium* using gas mixtures do not resemble the other measurements cited above   External diffusion rate is probably limiting in these experiments since at low CO tensions Q_{10} is unity, whereas in the experiments of Warburg and of Emerson (1936) with *Chlorella* using buffer mixtures Q_{10} is high

<p style="text-align:center">v</p>

General Considerations

It has been suggested (Hoover, Johnston, and Brackett, 1933, Brackett, 1935) that shading by the plastids may produce a gradation of light intensities at different plastids and thus affect the shape of the curve relating intensity and photosynthesis   While the light intensity is certainly not the same at all the different chlorophyll centers in the plant, it does not seem likely that the intensity photosynthesis relation is determined by such an effect, particularly since the curve is the same for many different species, and the size and number of chloroplasts must be very different for unicellular algae such as *Chlorella* and *Hormidium* and higher plants like wheat and *Cabomba*   The fact that Emerson's data for two widely different chlorophyll concentrations in *Chlorella* give the same curve, lends support to the idea that these curves represent some other mechanism than shading

The argument has also been advanced that the CO_2 photosynthesis curves may be affected by unequal CO concentrations at different photosynthetic centers   When diffusion rate limits photosynthesis, this is certainly true, but when CO_2 is supplied at a rapid rate this

situation probably does not occur   In those cases where diffusion is non-limiting, the curves relating photosynthesis with both CO_2 and intensity are identical   It does not seem likely that two such effects on different variables should produce identical equations

The effect of both CO_2 and intensity may be expressed in an equation of the type used by Baly (1934, 1935) and by Emerson and Green (1934 b), where p is the rate of photosynthesis and

$$p = k_1 I(a - x)^{\frac{1}{2}} = k_2 [CO_2] x^{\frac{1}{2}} \tag{10}$$
$$p = k_1 I(a^* - x^2)^{\frac{1}{2}} = k_4 [CO_2] x \tag{11}$$

a may be regarded as representing the total concentration of chlorophyll, and x the amount of chlorophyll activated by light   If x is eliminated and equation (10) or (11) is solved for p, equations are obtained relating p and either I or $[CO_2]$, which describe curves identical with that of equation (5)

Similarly, the equation

$$p = k_1 I(a - x)^{\frac{1}{2}} = k_2 [CO_2] x \tag{12}$$

with x eliminated and solved for p, yields curves identical with (7) It is assumed that CO_2 cannot enter in the same term as the light intensity, since this would result in low temperature coefficients at low CO_2 concentrations, which is not true when the external diffusion rate is non-limiting (Emerson and Green, 1934 b, Emerson, 1936)

The CO_2 does not appear to be bound by the unilluminated chlorophyll   If it were, the concentration of the CO_2-chlorophyll compound would be at a maximum after a period of darkness   The maximum rate of photosynthesis would then be obtained at the beginning of illumination   Actually the measurements of Warburg, 1920) (also see Baly, 1934) show that after a period of darkness the rate of photosynthesis slowly rises to a maximum indicating that the dark reaction follows the photochemical reaction

The above equations (10, 11, and 12) may be derived on the assumption that two reactions are involved in the cycle, a photochemical reaction during which light is absorbed, and a dark process which accomplishes a transfer of energy for the reduction of CO_2 The rate of photosynthesis (p) is equal to the rate of the dark reaction because this appears to be the reaction during which CO_2 is reduced and

oxygen is liberated   But there is apparently a third reaction which is involved in the cycle since CO_2 appears to be taken up in the dark by some protoplasmic constituent, as shown by Willstätter and Stoll It is not the purpose of the present paper to develop a kinetic scheme including this third reaction   This has already been considered by Briggs (1935) and others   We merely wish to indicate that equation (10) or (11) will give a quantitative description of the data relating rate of photosynthesis with CO_2 concentration and light intensity Including the third reaction will not change the properties of these equations but the interpretation   The velocity of the dark reaction will depend not on the CO_2 concentration directly but on the concentration of the CO_2-containing compound

The equations of Ghosh (1928), Emerson and Green (1934 b), Baly (1935), Burk and Lineweaver (1935), and Arnold (1935) describing photosynthesis as a function of intensity may all be put into the same form as equation (8) [1]   Ghosh, and Burk and Lineweaver used Harder's data, which have so high an experimental error that they are not critical   Baly used only the intensity data of Warburg and did not obtain a satisfactory agreement with them   Emerson and Green, and Arnold (1935) have not published any tests of their equations with the data of intensity and CO concentration   The fact that the data presented in this paper, both original and from others, do not fit equations derived by the above investigators provides a specific criticism of their equations

Arnold's kinetic scheme is based on studies made with intermittent illumination, which indicate that both the Blackman reaction (Arnold, 1933) and the photochemical reaction (Emerson and Arnold, 1932 b) are first order [3]

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[1] Since it is not the purpose of this paper to present a critique of the various kinetic schemes which have been suggested, the equations of these authors are considered together   It is realized that the various formulations differ in many important respects, but we are concerned here only with the quantitative treatment of the variables studied in this research

[2] Briggs has pointed out that equations similar to (8) are inadequate but does not give any quantitative test of his own scheme for photosynthesis rate as a function of I and $[CO_2]$

[3] We are not entirely satisfied with the assumptions inherent in both of these

It may be that the Blackman reaction is first order, as in equation (11) or (12), but the data relating intensity and photosynthesis are such that the photochemical reaction must be half order   However, by squaring the stationary state equation (10) we obtain

$$k_1^2 I^2(a - x) = k_2^2[CO_2]^2 x \tag{13}$$

which will describe the data if p remains proportional to $x^{\frac{1}{2}}$, as in equation (10)   Such a mechanism might be correct as it would yield first order photochemical and Blackman reactions, but I and $[CO_2]$ would now enter as the square   Emerson and Arnold also state that the yield per flash of light is independent of the intensity if the total energy per flash of light is constant, i e , the product of intensity and time is constant   From this it is concluded that I must enter as the first power   But the product of intensity and time could still be equal to a constant if both intensity and time were squared   It is difficult to understand why p should be proportional to $x^{\frac{1}{2}}$ in such a system, but it may be necessary if the findings of Arnold and of Emerson and Arnold are correct

The fact that photosynthetic rate is the same function of both CO_2 concentration and intensity is a simplifying feature of the kinetic scheme   Still, the presence of a fractional exponent or of intensity as the square indicates a complex system   There is no difficulty in accepting an equation in which CO_2 enters as the square, but in simple photochemical systems intensity enters as the first power, or in some reactions, such as those involving halogens, as the square root (Griffith and McKeown, p 407, 1929)   We are not aware of any photochemical reactions for which there has been accepted an equation in which I enters as a power above one   Nevertheless, such may

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proofs, and Emerson (1936) is likewise inclined to be skeptical of Arnold's proof of the first order character of the Blackman reaction

The evidence indicating that the light reaction is first order depends upon measurements made by varying the light intensity of short flashes of red light The total intensity range used was 1 to 10 or 1 log unit   Over this small range, the data are just as easily satisfied by assuming a half-order reaction   To be certain of the proof, it would be necessary to reinvestigate this problem over a satisfactory range of light intensities making certain that a condition of light saturation had been reached

be the case for photosynthesis and would perhaps indicate a chain process with more than one light reaction   This would be in keeping with the discovery of Warburg and Negelein (1923) that 4 quanta are necessary for the reduction of a single CO_2 molecule

Recent attempts to formulate a chemical mechanism for photo synthesis involve the postulation of several light reactions (Stoll, 1932, 1936, Willstatter, 1933, Franck, 1935)   Gaffron and Wohl (1936) have reviewed these efforts, and have shown that these 4 light reactions would have to be concurrent rather than consecutive Support for this idea has come from Kohn (1936) who has pointed out that 4 quanta could not be absorbed by the same chlorophyll molecule during a short light flash and still yield the amount of oxygen actually produced per flash

Such considerations indicate the necessity for a revision of our ideas concerning the cyclical process involved in photosynthesis   A scheme would have to be developed in which several light reactions take place concurrently with the absorption of quanta by different chlorophyll molecules   Subsequent dark reactions would restore the chlorophyll to its original condition, and the energy released used for the reduction of CO_2 and water   However, for a description of the data of CO_2 and intensity, the simple two part cycle appears to be adequate, provided that the equation has the exponents given above   The accumulation of more kinetic data will determine the further utility of the two-reaction cycle

SUMMARY

1   An optical system is described which furnishes an intensity of 282,000 meter candles at the bottom of a Warburg manometric vessel With such a high intensity available it was possible to measure the rate of photosynthesis of single fronds of *Cabomba caroliniana* over a large range of intensities and CO_2 concentrations

2 The data obtained are described with high precision by the equation $KI = p/(p^2_{max} - p^2)^{\frac{1}{2}}$ where p is the rate of photosynthesis at light intensity I, K is a constant which locates the curve on the I axis, and p_{max} is the asymptotic maximum rate of photosynthesis   With CO_2 concentration substituted for I, this equation describes the data of photosynthesis for *Cabomba* as a function of CO_2 concentration

3 The above equation also describes the data obtained by other investigators for photosynthesis as a function of intensity, and of CO_2 concentration where external diffusion rate is not the limiting factor This shows that for different species of green plants there is a fundamental similarity in kinetic properties and therefore probably in chemical mechanism

4 A derivation of the above equation can be made in terms of half-order photochemical and Blackman reactions, with intensity and CO_2 concentration entering as the first power, or if both sides of the equation are squared, the photochemical and Blackman reactions are first order and intensity and CO_2 enter as the square   The presence of fractional exponents or intensity as the square suggests a complex reaction mechanism involving more than one photochemical reaction This is consistent with the requirement of 4 quanta for the reduction of a CO_2 molecule

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THE INFLUENCE OF LIGHT ADAPTATION ON SUBSEQUENT DARK ADAPTATION OF THE EYE*

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I

Rod and Cone Adaptation

Human dark adaptation, though described by Aubert in 1865 and first measured by Piper in 1903, is still inadequately known. The early data of Piper seemed to show that dark adaptation was an exclusive function of the rods. Only after the measurements of foveal cone adaptation had been made (Hecht, 1921) was the reason for this apparent cone adaptation is so fast that Piper missed it completely. The confirmation and extension of these results by Kohlrausch (1922) emphasized the existence of both cone and rod dark adaptation, and showed that the two are to a certain extent sharply separated in time.

Following adaptation to ordinarily bright lights, dark adaptation occurs in two parts. The first begins at once, it is rapid, and is due to cone function. The second part shows up somewhat later, it is slow, and is due to rod function. Under these circumstances, cone adaptation is over in 3 or 4 minutes, whereas rod adaptation takes at least 30 minutes. The intensity range covered by the rods and by the cones during dark adaptation depends on the color of the measuring light (Kohlrausch, 1922, 1931), on its area and retinal location (Hecht, Haig, and Wald, 1935), and on the duration and intensity of the preceding light adaptation (Müller, 1931, Wald and Clark, 1936, Winsor and Clark, 1936, Hecht and Haig, 1936).

In this paper we shall describe in detail the effects of the intensity of light adaptation on the dark adaptation which follows it, and show what bearing this information has on other data of visual function

II

Apparatus and Method

There are five special points in our arrangements for making the measurements   First, the eye is light adapted with the observer in place so that measurements of dark adaptation can begin immediately   Second, the optical system gives a constant pupil size without the inconvenience of an artificial pupil   Third, violet light is used for measurement to secure the largest range of rod adaptation, and red light to secure the smallest   Fourth, each exposure to the measuring light is a flash of 0 2 second   Fifth, the measurements represent not quite the threshold of vision but a brightness about 3 times as high, secured by using the appearance of a black cross as criterion

The data obtained in this way are only slightly more accurate than those usually made with a diffuse threshold criterion, indefinite time for determining the threshold, an artificial pupil as ordinarily used, and even without an artificial pupil at all   However, the arrangements make the work easier for the observer, and give him a feeling of certainty in making the measurements

The observer sits in a dark box in a dark room, with his head fixed in a chin and head rest, facing a fixation point which is so circumscribed by diaphragms as to place his eye with precision at a given spot in space   This spot is at the focus of two lenses, one for light adaptation and the other for dark adaptation

The source for light adaptation is a 3 8 volt flash-light bulb running on 0 3 amp , and placed 9 cm from a pair of 11 diopter lenses 5 cm in diameter each   The arrangement is shown in the lower right of Fig 1   The eye is placed at the conjugate focus at an equal distance on the other side of the lenses, and sees the nearer lens evenly illuminated as a large field 30° in diameter   Its brightness is 400,000 photons, as determined by a binocular photometric match   In front of the lens, filters may be inserted to reduce the brightness   To save space a mirror is introduced between the lamp and the lens   In these experiments light adaptation is always with whole (white) light

The light adaptation arrangement is mounted on a small board sliding on rails, and can be moved into the dotted position shown in Fig 1 in front of the eye   At the close of light adaptation, it can be instantly slid out of the way so

as to permit view of the second lens which focusses the measuring light on the same spot just inside the cornea of the eye at the pupil

The light for measuring dark adaptation is a 3 8 volt flash light lamp, carefully maintained at 0 28 amp , and placed 31 cm from a 6 5 diopter lens, 3 6 cm. in diameter   The pupil of the eye is at the conjugate focus, at an equal distance on the other side of the lens, so that the lens appears as an evenly illuminated field   The image of the filament produced by the lens is less than 1 sq mm , and therefore falls entirely within even the smallest dimensions assumed by the pupil of the eye   This arrangement avoids the necessity for an artificial pupil to keep
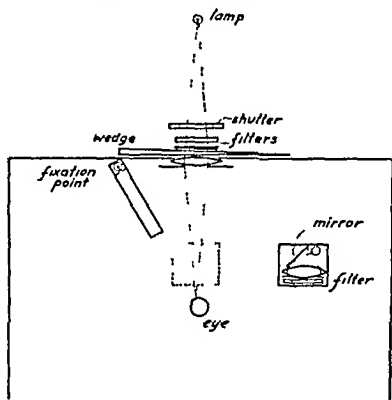
FIG 1 Arrangement for adapting the eye to different intensities of light, and for measuring the course of the subsequent dark adaptation   In the set up for light adaptation in the lower right only one lens has been drawn in, actually there are two

constant the area of the beam as it enters the eye   A circular diaphragm cuts the lens to form a field whose diameter is 5° visual angle   Extending over the entire field is an opaque cross, the width of whose arms occupies a visual angle of 30′ and thus corresponds to a visual acuity of 0 033, which is perceptible almost at the threshold of vision   The intensity of the measuring light is controlled by decimal neutral filters manipulated by the recorder, and by a neutral wedge moved by the observer   The color is secured either with a violet filter (Corning No 511) which transmits light below 480 mμ only, or with a red filter (Wratten No 88) which transmits light above 690 mμ only   The exposure is made with a photo

graphic shutter by a cable in the hands of the observer The arrangements are shown diagrammatically in Fig 1

The center of the light-adapting field and that of the measuring field are at 30° nasally on the retina Note that the light-adapted area is 36 times the size of the measuring area The fixation point is central, of course Its intensity is varied by a rheostat controlled by the observer so as to be adequate for fixation but not too bright

Light adaptation is for precisely 2 minutes It is always preceded by a 10 to 15 minute stay in the dim light of the dark room in order to remove the effects of the variable conditions to which the observer has been exposed Measurements are made at definite intervals after light adaptation, the observer determining the minimum brightness at which he can just see the black cross silhouetted against the field He does this by moving the wedge to a position below the threshold, then turning it up in steps, and testing at each step by a flash until he can see the cross The observer allows at least 20 seconds and frequently more between flashes in making a determination In the first 2 minutes after light adaptation, dark adaptation is so rapid that a slightly different procedure is required The wedge is brought to a given position which is maintained until the observer, who tests for its visibility at 10 or 15 second intervals, announces that he can see the field

<div align="center">III</div>

<div align="center">

Measurements with Violet Light

</div>

Our measurements were made with the right eye only, and we three served as observers   S H and A M C are emmetropes, C H is a myope, and used a minus 6 diopter lens in front of his eye as a correction   Five intensities of light adaptation were chosen after preliminary trials in order to cover the main range of the phenomenon Each observer made two runs for each light adaptation and for each color   The data in Table I are the averages of the two runs using violet light for measurement   The intensities are in microphotons, that is, millionths of a photon   A photon is the retinal illumination produced when the eye looks through a 1 sq mm pupil at a surface whose brightness is $\pi/10$ millilamberts   The individual runs are nearly as regular and precise as the averages, but we use the averages because of day to day variations in threshold, the data are thus more homogeneous as a whole   The measurements for S H are shown graphically in Fig 2   The data for the other two observers are the same in all essentials, and Fig 2 will serve as a description for them as well

TABLE I

Dark adaptation measured with violet light following adaptation to different intensities of white light Time is in minutes intensity in microphotons The heavy values show color at the threshold

Observer	400 000 photons		35 900 photons		19 500 photons		3 800 photons		263 photons	
	Time	Log I	Time	Log I	Time	Log I	Time	Log I	Time	Log I
S H	0 19	8 26	0 10	7 16	0 17	6 96	0 18	6.22	0 14	4 78
	0 52	7 58	0 57	6 53	0 42	6 41	0 42	5 60	0 36	4 38
	1 1	7 07	1 0	6 11	0 97	6 11	0 67	5 25	0 50	4 12
	1 5	6 80	2 5	5 90	1 7	5 94	1.3	4 92	0 90	3 88
	2 2	6 37	3 3	5 81	2 7	5 75	2 1	4 72	1 4	3 71
	2 7	6 19	4 1	5 76	4 1	5 59	2 9	4 54	2 9	3 57
	3 4	6 00	5 3	5 75	5 1	5 42	3 8	4 41	4 1	3 45
	4 4	5 92	6 1	5 67	6 3	5 17	4 9	4 15	5 3	3 33
	6 4	5 80	7 1	5 61	7 6	4 81	5 9	3 96	6 2	3 24
	7 7	5 73	7 8	5 45	9 2	4 38	7 7	3 60	7 2	3 07
	9 5	5 68	8 9	5 26	10 7	3 98	9 4	3 40	8 9	3 02
	10 7	5 66	9 9	4 99	11 9	3 70	10 6	3 16	10 5	2 91
	12 6	5 34	10 8	4 77	13 0	3 50	13 5	2 89	11 5	2 82
	14 3	4 78	12 5	4 28	14 4	3 28	15 0	2 80	13 1	2 80
	16 0	4 28	14 3	3 81	16 1	3 07	16 4	2 77	15 1	2 72
	16 7	4 11	15 4	3 5?	17 8	2 92	18 2	2 62	17 3	2 61
	18 0	3 79	16 8	3 29	18 8	2 83	21 9	2 56	19 7	2 58
	19 6	3 55	18 6	3 13	20 0	2 79	23 7	2 49	23 5	2 51
	21 5	3 27	20 5	2 99	21 1	2 78	25 4	2 46	25 8	2 55
	23 0	3 20	22 5	2 92	24 5	2 64	28 8	2 47	27 5	2 51
	24 2	3 13	23 9	2 88	26 1	2 57			29 6	2 53
	25 9	3 08	26 0	2 79	27 7	2 57				
	28 5	2 97	29 3	2 69	29 8	2 58				
	30 8	2 88	32 0	2 55						
	33 4	2 84								
	36 0	2 78								
	38 6	2 72								
C H.	0 08	8 26	0 19	7 06	0 12	6 87	0 19	6 08	0 12	4 91
	0 38	7 12	0 66	6 47	0 42	6 26	0 64	5 28	0 30	4 45
	0 65	6 65	1 3	6 05	0 74	6 00	1 2	5 08	0 74	3 97
	1 2	6 31	1 8	5 86	1 4	5 87	2 0	4 82	1 7	3 80
	2 3	5 99	2 8	5 76	2 3	5 75	2 5	4 70	2 4	3 57
	3 1	5 78	4 3	5 59	3 2	5 66	3 3	4 59	3 3	3 42
	4 0	5 64	5 4	5 57	4 3	5 54	4 3	4 45	4 5	3 37
	4 8	5 57	6 3	5 54	5 2	5 40	5 6	4 30	5 6	3 22
	5 7	5 55	7.5	5 50	5 9	5 17	6 8	4 06	7 0	3 11
	7 8	5 54	8 1	5 27	6 8	4 98	7 9	3 82	8 1	3 02
	10 5	5 48	9 2	5 02	7 9	4 68	9 2	3 67	9 8	2 98
	12 4	5 46	10 3	4 64	9 0	4 45	10 4	3 51	10 7	2 93
	13 5	5 41	11 7	4 42	11 0	4 05	11 8	3 37	12 2	2 92

TABLE I—*Concluded*

Following light adaptation to 400,000 photons, dark adaptation as measured with extreme violet light shows a striking separation into two sections, obvious in Fig 2   Judging by all that is already known about dark adaptation (Kohlrausch, 1931, Hecht, Haig, and Wald, 1935), intensity discrimination (Hecht, 1935), and flicker (Hecht and Shlaer, 1936), the first section must be identified as predominantly cone adaptation and the second section as predominantly rod adaptation   This identification is immensely strengthened by the
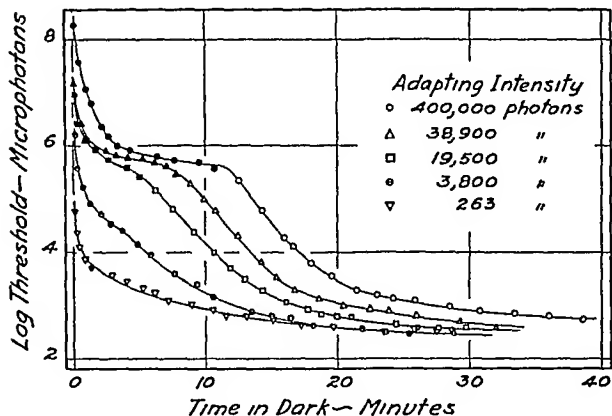
Fig 2 The course of dark adaptation as measured with violet light following different degrees of light adaptation   The filled in symbols indicate that a violet color was apparent at the threshold, while the empty symbols indicate that the threshold was colorless

fact that all the measurements comprising the first section are reported by the observer as distinctly showing a violet color at the threshold of observation, whereas all the measurements comprising the second section are reported as colorless at the threshold   The points reported as colored are shown as black circles in Fig 2 and are printed in heavier type in Table I, while those reported as colorless are the clear circles in Fig 2   The transition between colored primary

section and the colorless secondary section is quite sharp, occasionally the last colored point is less saturated than the others

This description of the events in dark adaptation holds also for the measurements which follow light adaptation to 38,900 and to 19,500 photons   In all three, cone and rod adaptation are separate and are distinctly associated with colored and colorless sensations at the threshold   There are, however, certain obvious quantitative differences   As the light adaptation intensity increases, so also does the range of cone dark adaptation   Following the highest light adaptation, the cone dark adaptation range is about 3 log units, which compares favorably with the rod adaptation range   The range of rod dark adaptation, however, remains unchanged, but its appearance is more delayed the higher the light adaptation   This is an important point, and is also apparent in Muller's measurements in which he varied the duration of the preceding light adaptation

After 263 photons light adaptation, the dark adaptation threshold never shows any color   A M C occasionally records the first point as possibly colored   On the other hand, dark adaptation following exposure to 3,800 photons shows a course which is midway between high and low light adaptation   The first few measurements are definitely reported as violet, and are indicated as such by the use of filled-in symbols in Fig 2   After a few minutes, the threshold becomes colorless and the transition between the two is fairly gradual

The color response in this case is not too certain near the transition, but above it it is quite clear   What is startling, however, is that color is definitely associated with threshold intensities well below those which show no color following adaptation to the high intensities   We do not know what this means, and whether it is a special property of violet light, we are therefore investigating other colors as well

IV

Displacements in Time

Looking at Fig 2 as a whole, it is apparent that the higher the initial light adaptation, the longer does the eye require to reach a given threshold during dark adaptation   This is true for the colored primary portion as well as for the colorless secondary portion, but shows more strikingly for the latter   For the three high light adapta-

tions, where the range of the colorless portion is the same, it is as if the rod curve as a whole were moved so as to appear later the higher the light adaptation

In order to compare the time of appearance of the two sections at the different adapting intensities, we have determined the time in the dark which has to elapse for the eye to reach a specific threshold intensity level following each of the light adapting intensities   For the colored primary portion, the level selected is $\log I = 6\ 25$, while for the colorless secondary portion the level selected is $\log I = 3\ 75$ The determinations were made graphically from the data in Table I They are given in Table II and are shown graphically in Fig 3, where

Relation between intensity of adapting light and time of appearance of a specific threshold during dark adaptation   Threshold for colored portion is $\log I = 6\ 25$, threshold for colorless portion is $\log I = 3\ 75$ Time is in minutes

Adaptation Intensity	Colored portion			Colorless portion		
	S H	C. H.	A M C.	S H	C. H	A M C.
photons						
263				1 4	1 7	0 5
3 800	0 20	0 18	0 20	6 9	8 5	7 6
19 500	0 65	0 35	0 55	11 7	12 1	13 5
38 900	0 75	0 90	1 10	14 5	16 0	16 3
400 000	2 45	1 45	1 80	18 3	22 5	20 3

the colored portion is in the upper, smaller figure, while the colorless portion is in the lower, larger figure

It is clear that the colorless secondary portion appears later as the adapting light intensity is increased, the relation between the time of appearance and the logarithm of the adapting intensity is sigmoid

The measurements for the colored primary portion show that for it too the time in the dark required to arrive at a specific threshold increases with the logarithm of the adapting intensity   However, whereas the relationship for the colorless rod section is nearly completely covered by the range of intensities used, the relationship for the colored cone section begins to be effective only at the higher light adaptations   It is likely that the relation here would also be sigmoid if we explored adapting intensities still higher than those used

The meaning of these shifts in the time of appearance of a given

threshold with the intensity of the adapting light is fairly clear
Blanchard (1918) has shown that as the light adapting intensity
increases, the threshold at zero time in the dark also increases   Dark
adaptation therefore has a larger range to cover, and a given threshold
will be reached later and later   To a certain extent, it is as if the
curve of dark adaptation for each portion were shifted to the right on
the time axis   Since the rods and cones are different sensory systems
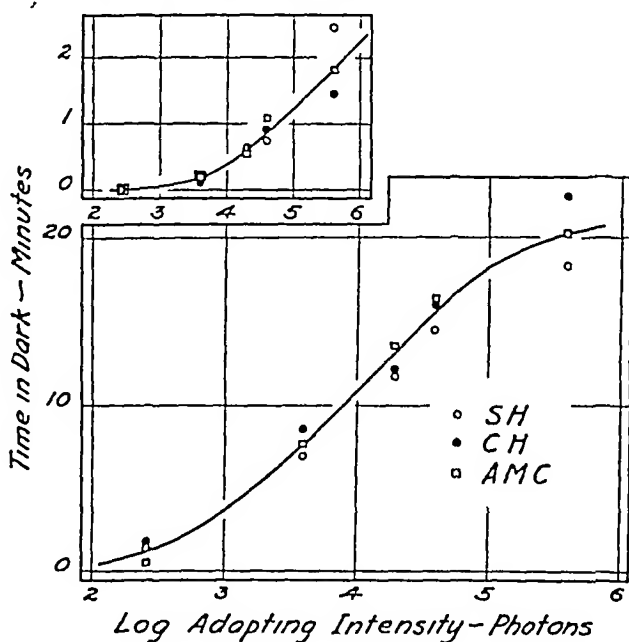
FIG 3 The relation between the intensity of light adaptation and the time
required for the eye to reach a given threshold in the dark   The lower data are
for a colorless threshold, indicating rod function, while the upper data are for a
color threshold indicating cone function

with different sensibilities and different rates of dark adaptation, it is
not surprising that the amount of shift is different for each system
    Fig 3 shows that the time displacements for the cones are short,
of the order of 1 or 2 minutes, while for the rods they are about 10
times as long   The time for complete dark adaptation of the two
systems also differs in about the same ratio   cone adaptation is nearly
complete in 4 or 5 minutes, while rod adaptation takes about 10 times
as long   Thus the time shifts shown by the cones and by the rods are
about the same fractions of the total adaptation time for each

V

Similarities among Individuals

Dark adaptation has usually been considered a fairly variable property of vision, and Matthey (1932) has felt it necessary to meas ure over 50 people in order to arrive at an average or standard curve of dark adaptation useful for clinical purposes   In our experience of over 10 years, we have found that most of the variations in the data arise from the failure to specify conditions of measurement   The major differences may be eliminated by fixing the intensity and dura tion of light adaptation, and the position, area, and color of the light used for measuring dark adaptation

With all these specified, there is nevertheless a slight day to day variation even in a single experienced investigator   The final thresh old varies and the time of appearance of the two parts of adaptation varies   What is specially significant is that the variations may be different in sign and in extent for the two parts of dark adaptation

In spite of these slight differences, the shapes of the data are iden tical at different times for the same observer, and even for the three observers   For example, although the time of appearance of the rod curve following the highest light adaptation differs by about 2 minutes for A M C and S H , nevertheless the rod curves for the two investi gators are identical if the data of one are shifted 2 minutes along the time axis   To show this, we have superimposed in Fig 4 the data for the three observers, but have kept the colored and colorless portions of the data separate   We have included in Fig 4 only the highest three light adaptations, because in them the two sections of the subsequent dark adaptation are easily separable   For each person there has been made the necessary time shift, and whenever required, a small vertical intensity shift   It is apparent that the three observers show identical curves under these circumstances

Because of the individual differences in time of appearance of the transition from cone section to rod section, a simple averaging of data of different individuals is bound to yield an erroneous appearance of the dark adaptation curve   The region of transition, from being a reasonably abrupt change for each individual, will become smooth and gradual in the average, and may indeed almost disappear   This is

actually the case with Matthey's average curve for 54 individuals, which shows almost no sign of transition, though the measurements were made with the apparatus and specified conditions worked out by Muller (1931) whose measurements on his own eye clearly show the usual fairly abrupt transition (*cf* Hecht, 1937 *a*) If it is necessary to have an average dark adaptation curve for clinical purposes, the cone sections and the rod sections should be averaged separately, and
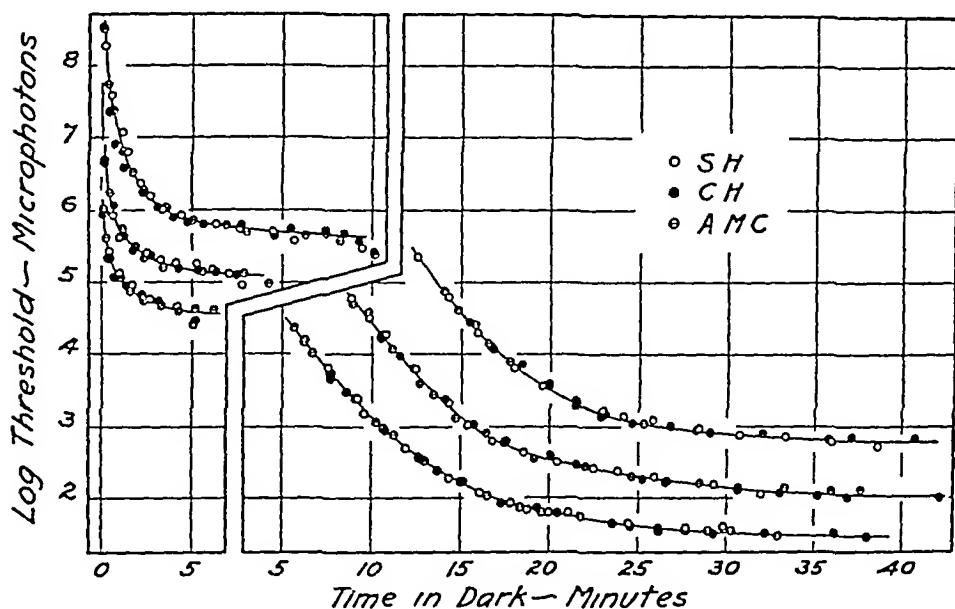
Fig 4 Dark adaptation following the highest three intensities of light adaptation The colored cone thresholds are plotted separately from the colorless rod thresholds The abscissas are the same for all the data, the ordinate scale, however, applies only to the upper data because the two other sets have been displaced 0 5 and 1 0 log units respectively downward

the two combined at a point which is the average transition time for the same observers

The curves drawn through the cone portions in Fig 4 are the same for the three adapting intensities By making the time shifts indicated in Fig 3 the data themselves may be superimposed, showing that they fall on the same curve of which more and more becomes available as the light adaptation intensity increases The three curves for the colorless portions in Fig 4 are very nearly but not quite the same The slope of the rising branch becomes just percep-

tibly faster the higher the light adaptation   However, the difference
is so slight that it is possible to superimpose the three sets of points,
and to draw one curve through them   Apparently then, above a
light adaptation intensity of 20,000 photons, the subsequent dark
adaptation has a fixed shape but a variable position on the time axis

<div align="center">VI</div>

Measurements with Red Light

In terms of the visibility curves of cone vision and rod vision (*cf*
Hecht, 1937 *a*), we may expect the difference in threshold between
rods and cones to be greatest with violet light, and least with red
light   In fact, with extreme red light (Hecht, 1921, Kohlrausch,
1922, 1931) the difference in threshold may disappear entirely, so
that the resulting dark adaptation curve records only the more
rapidly acting cone function

Using such extreme red light we repeated our measurements of dark
adaptation following different intensities of light adaptation   The
data are in Table III, and are the averages of two runs for each of the
three observers   We used only four intensities of light adaptation,
the fifth was too low to cause any dark adaptation measurable with
red light

Fig 5 shows the measurements of A M C and brings to light
several interesting things   The data behave much like the primary
cone sections with violet light shown in Fig 2, except that the red
threshold remains at the level it reaches after the first 5 minutes
Clearly this is pure cone adaptation   Nevertheless, even with red
light, rod adaptation makes its appearance   Following light adapta
tion to 400,000 photons, the threshold during dark adaptation is red
in appearance for over 35 minutes   This is shown by the solid circles
in Fig 5   After 35 minutes there appears a slight drop in threshold,
and this new threshold is characterized by being either very dilute
pink or quite colorless (open circles)   Moreover, just as with violet
light, the drop in threshold and the loss of color come sooner the lower
the intensity of preceding light adaptation   Fig 5 shows this quite
clearly and leaves no doubt that the change records the belated appear
ance of the bottom of the rod adaptation curve

TABLE III

Dark adaptation measured with red light following adaptation to different intensities of white light. Time is in minutes, intensities in microphotons

Observer	400 000 photons		38,900 photons		19,500 photons		3,800 photons	
	Time	Log I	Time	Log I	Time	Log I	Time	Log I
S H	0 55	7 87	0 28	7 85	0 15	7 63	0 08	6 93
	1 0	7 15	0 53	7 44	0 40	7 14	0 25	6 50
	1 7	6 71	1 1	6 91	0 68	6 97	0 48	6 25
	2 3	6 34	1 7	6 63	1 3	6 56	0 99	6 07
	2 9	6 22	2 3	6 38	1 8	6 34	3 0	6 03
	3 8	5 98	3 0	6 12	2 9	6 00	4 3	5 94
	4 5	5 89	3 9	5 98	4 7	5 99	7 4	5 90
	5 7	5 86	5 2	6 01	9 3	6 04	9 5	5 92
	7 1	5 84	6 5	5 99	11 5	5 98	11 4	5 89
	9 0	5 81	10 3	6 00	13 7	5 98	13 0	5 87
	10 8	5 83	14 0	6 01	16 6	5 92	15 4	5 86
	14 4	5 86	16 1	5 97	19 4	5 93	19 9	5 79
	16 9	5 76	18 0	5 94	21 6	5 87	24 0	5 77
	18 5	5 80	20 7	5 92	24 5	5 80	27 0	5 78
	20 0	5 79	22 2	5 93	27 5	5 76	30 6	5 75
	21 6	5 76	24 9	5 91	30 6	5 77	34 7	5 75
	23 9	5 83	29 6	5 89	33 1	5 68		
	27 7	5 76	32 5	5 84	36 4	5 67		
	29 6	5 77	37 5	5 78				
	33 3	5 78						
	36 0	5 75						
	39 5	5 79						
C H	0 36	7 85	0 14	7 63	0 07	7 37	0 10	7 28
	0 90	7 48	0 41	7 14	0 35	6 64	0 48	6 50
	1 3	7 27	0 81	6 73	0 93	6 28	0 83	6 38
	2 0	6 85	1 5	6 21	1 7	6 11	1 9	6 25
	3 0	6 54	2 9	6 05	3 0	6 05	3 5	6 18
	4 2	6 22	4 2	5 98	4 0	6 00	4 9	6 19
	5 4	6 09	7 6	6 03	5 8	5 97	6 6	6 13
	6 8	6 06	9 0	6 02	8 5	6 09	9 2	6 10
	9 0	6 05	10 6	6 03	10 9	6 02	14 5	6 06
	10 9	6 04	14 2	6 01	14 2	6 00	20 2	5 83
	14 8	6 08	18 1	6 06	21 2	5 86	22 0	5 77
	16 2	6 04	21 0	6 02	23 0	5 83	27 5	5 77
	20 6	6 07	26 1	6 04	26 4	5 82	30 8	5 73
	26 8	6 17	31 1	5 98	30 5	5 79		
	33 0	6 22	36 8	5 98	33 8	5 77		

TABLE III—*Concluded*

Observer	400 000 photons		35 900 photons		19 500 photons		3,800 photons	
	Time	Log I	Time	Log I	Time	Log I	Time	Log I
A. M C	0 30	7 85	0 15	7 71	0 19	7 28	0 09	6 58
	0 60	7 33	0 35	7 27	0 35	6 96	0 36	6 10
	0 93	7 02	0 76	6 71	0 80	6 57	0 81	5 78
	1 4	6 69	1 2	6 48	1 1	6 36	2 2	5 68
	1 8	6 41	1 7	6 26	1 5	6 22	3 2	5 63
	2 6	6 12	2 5	5 91	1 9	6 04	4 4	5 63
	3 4	5 88	3 5	5 78	2 6	5 86	6 1	5 62
	4 4	5 81	5 2	5 73	4 0	5 73	7 0	5 54
	5 9	5 72	6 8	5 66	5 8	5 67	8 0	5 46
	7 6	5 70	8 2	5 62	6 7	5 67	8 8	5 49
	8 6	5 60	12 8	5 69	8 1	5 67	11 9	5 48
	10 2	5 61	14 3	5 69	9 3	5 63	14 2	5 43
	12 5	5 66	16 6	5 65	11 2	5 66	15 8	5 29
	14 1	5 66	19 4	5 60	12 6	5 60	18 4	5 21
	17 3	5 66	22 1	5 61	14 2	5 56	21 4	5 22
	19 4	5 66	26 1	5 56	16 4	5 59	23 8	5 23
	24 9	5 67	28 3	5 55	17 9	5 61	27 2	5 19
	27 4	5 64	30 6	5 55	19 9	5 54	28 8	5 16
	31 7	5 64	33 0	5 55	21 0	5 54	31 4	5 16
	37 0	5 56			22 0	5 51		
	40 0	5 57			24 6	5 50		
					27 1	5 52		
					30 5	5 43		
					33 1	5 48		

To the first three curves in Fig 5, we have added as crosses the cone portions of the measurements with violet light made by A M C   It is apparent that they correspond reasonably well with the red light measurements, and thus furnish additional evidence of their character as cone function   After the highest light adaptation the violet light measurements tend to drop below the red light measurements as the former approach the transition point for rod appearance

As with the cone portions of the violet light data, the same curve may be drawn through the three sets   It is merely its vertical extent and its position on the time axis which change   The displacement along the time axis is also much as with the violet light data In fact the average shift plotted against the logarithm of the adapting

intensity has precisely the same slope and appearance as the violet light data in Fig 3   All this confirms the conclusion that the two series of measurements record the same phenomenon, namely cone adaptation
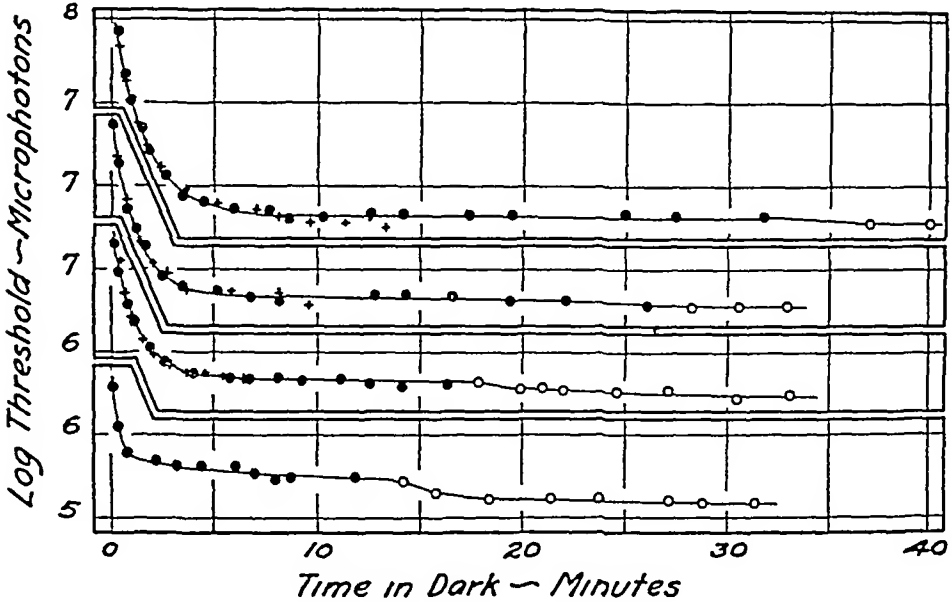
FIG 5 The course of dark adaptation as measured with red light following different degrees of light adaptation   Here too the filled-in symbols indicate that the threshold was colored red, and the empty symbols indicate that the threshold was colorless

<div align="center">VII</div>

Two Types of Rod Adaptation

These data are of theoretical interest in a way that has already been emphasized by Winsor and Clark (1936) and by Wald and Clark (1936) from a different point of view   Rod dark adaptation apparently can follow two different courses depending upon the intensity of the light used for preadaptation   Fig 2 shows that following low intensities of light adaptation, rod dark adaptation begins at once and changes its rate so that the points leave the log I axis gradually and approach the final threshold on the time axis asymptotically   Following high intensities of light adaptation, rod dark adaptation

does not show itself at once, and when it does appear, its speed seems to remain roughly constant for some time so that its curve on a t log I plot begins in an almost linear fashion   It leaves the log I axis sharply and only after several minutes does it begin to slow up and approach the t axis asymptotically   We may refer to these two kinds of rod dark adaptation as the rapid type and the delayed type   Data at intermediate intensities present a combination of the two, in Fig 2 showing the data for S H , the two are just apparent even in the lowest curve

To judge by these two courses of dark adaptation, it is justifiable to conclude, as have Winsor and Clark, and Wald and Clark, that the accumulation of sensitive material, which dark adaptation undoubt edly represents, may occur in two ways   It is important to recognize precisely when these two types of adaptation appear   Examination of Fig 2 shows that the delayed type is definitely established above 20,000 photons, and that the rapid type is clearly established below 200 photons   Between these two values, the adaptation shows both characteristics

It is significant that even the lowest preadaptation intensity here used is many times higher than that required to achieve a maximum effectiveness of the rods in all the other functions in which they have been studied   For example, the maximum flicker frequency shown by the rods is achieved between 0 1 and 1 photon (Hecht and Smith, 1936)   Similarly, the maximum visual acuity attained by the rods is also apparent below 1 photon (cf Hecht, 1937 a) and so is the maximum intensity discrimination of the rods (Hecht, 1935), and the maximum instantaneous threshold (Blanchard, 1918, Hecht, 1937 b)   In other words, at a stationary state produced by a retinal illumination of 1 photon, the visual functions of the rods are at their maximum   Therefore the delayed type of dark adaptation first appears only after adaptation to intensities 200 times as great as those which are known to produce the maximum visual effectiveness in the rods and becomes established only at intensities 20,000 times as great Thus, what we call dark adaptation following preadaptations above 20,000 photons is probably a different phenomenon than dark adapta tion following preadaptations below 200 photons, though in both cases the same sensitive material, visual purple, accumulates in the rods

Kuhne (1879) first showed that visual purple in the retina may be regenerated by two methods after being bleached from the products of decomposition, and *de novo*   This has been confirmed and developed by Wald (1935 *a*, *b*) in a series of excellent researches with various retinas, moreover, Wald has been able to identify components of this visual cycle as vitamin A and a carotenoid, retinene, and has shown that retinene is the first product of bleaching in the retina and becomes converted later into vitamin A   We have recently been able to regenerate visual purple in solution from the products of its bleaching by light (Hecht, Chase, Shlaer, and Haig, 1936)

It is quite likely that the two modes of formation of visual purple have different courses and different velocities   Wald and Clark (1936) have suggested that the faster course is the regeneration by way of photoproducts, and the slower by way of fresh substances from the retina or from the blood stream   These would then correspond to the rapid and the delayed types of dark adaptation which we find here We are now engaged in measurements which will formulate this correspondence more precisely

SUMMARY

The course of dark adaptation of the human eye varies with the intensity used for the light adaptation which precedes it   Preadaptation to intensities below 200 photons is followed only by rod adaptation, while preadaptation to intensities above 4000 photons is followed first by cone adaptation and then by rod adaptation

With increasing intensities of preadaptation, cone dark adaptation remains essentially the same in form, but covers an increasing range of threshold intensities   At the highest preadaptation the range of the subsequent cone dark adaptation covers more than 3 log units

Rod dark adaptation appears in two types—a rapid and a delayed The rapid rod dark adaptation is evident after preadaptations to low intensities corresponding to those usually associated with rod function The delayed rod dark adaptation shows up only after preadaptation to intensities which are hundreds of times higher than those which produce the maximal function of the rods in flicker, intensity discrimination and visual acuity   The delayed form remains essentially

constant in shape following different intensities of preadaptation However, its time of appearance increases with the preadaptation intensity, after the highest preadaptation, it appears only after 12 or 13 minutes in the dark

These two modes of rod dark adaptation are probably the expression of two methods of formation of visual purple in the rods after its bleaching by the preadaptation lights

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DO MELANOPHORE NERVES SHOW ANTIDROMIC RESPONSES?

BY G. H. PARKER

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Antidromic responses and axon reflexes both imply that under experimental conditions or even under certain unusual natural conditions nerve impulses may flow over nerve fibers in a direction the reverse of the usual one. This is perhaps most clearly seen in the motor system. The axon of a vertebrate motor neurone, as it approaches and enters its muscle, branches many times till its final ramifications end each in a cross-striped muscle fiber. The ultimate branches of such an axon may number a hundred or so and with each branch carrying at its end a muscle fiber the whole system collectively constitutes what Sherrington has called a motor unit. Such a unit is believed to act as a whole in the ordinary contraction of a skeletal muscle and under usual circumstances it represents the smallest functional aggregate of active muscular elements. The contraction of any vertebrate skeletal muscle is made up of the combined contractions of its various motor units each of which represents an irreducible element in this operation. If instead of stimulating the single motor axon of a given motor unit, the stimulus is applied to one of the peripheral branches of this axon, an interesting condition presents itself. Under such circumstances not only do the muscle fibers supplied by the stimulated branch contract, but those in the rest of the unit also shorten, showing that from the point of stimulation impulses not only flow peripherally in the normal direction into the attached muscle fibers but impulses also pass centrally, that is, antidromically, till they meet other peripheral branches whereupon they make their way peripherally to the other muscle fibers of the unit. An antidromic or backward flowing of impulses is quite consistent with what is known of general nerve physiology within the limits of a single

neurone and affords the basis for the so called axon reflexes   If on stimulating an axon branch of a motor neurone there is this antidromic flow of impulses, may we not expect a similar condition in vertebrate melanophore nerve fibers?   This question, which was raised very forcibly by Hoagland in his discussion of my recent paper on "The reaction of chromatophores as evidence for neurohumors" (Parker (1936 a)) given at the Cold Spring Harbor Symposium for 1936, is the subject of the present communication

The melanophore system upon which it is convenient to test this question is that of the common killifish, *Fundulus heterochtus*   This system, however, is not so simple as the skeletal motor system of vertebrates   In *Fundulus* as in other teleosts each melanophore receives several nerve fiber branches instead of only one as with crossed-striped muscle fibers and of these branches at least one is concentrating in function inducing a central migration of the melanophore pigment and at least one other is dispersing in function bringing about a scattering of the pigment   These melanophores, then, unlike skeletal muscle fibers, have a double innervation and this must be kept in mind in the following discussion

It has been known for over a decade (Wyman (1924)) that when a bundle of melanophore nerve fibers is cut in the tail of a pale *Fundulus*, the melanophores thus denervated form a dark band leading from the cut toward the periphery of the tail   This phenomenon has been shown (Parker (1934)) to be due to an excessive stimulation of the dispersing nerve fibers and may persist for several days   Many students of this subject have recorded the striking appearance of this band peripheral to the cut, but no one, so far as I am aware, has made any comment on its possible extension in a central direction from the cut   Such an extension would be indicative of antidromic action and from the analogy with skeletal muscle it should be expected to occur   The first question to be answered is whether there really is any evidence of this central extension?

When a single branched fin-ray in the caudal fin of a very pale *Fundulus* is severed a few millimeters distal from the root of the tail, the dark band already noticed begins to form within half a minute and is very fully developed in the course of 5 minutes   A most careful scrutiny with the unaided eye of the region of the cut after

the band has reached full formation reveals a well defined dark area that starts exactly at the cut and reaches peripherally to near the edge of the tail, but no band extends centrally from the cut A microscopic examination of these regions, however, shows a slightly different condition The position of such a cut is shown in Figs 1 and 2 (4) and a view of the cut itself as seen under the microscope is given in Fig 4 As is shown in Fig 1 each ray starts at the base of the tail as a single bony axis A short distance from this point it divides into two, then farther on into four and finally as a rule near the margin of the tail into eight subrays The cut shown enlarged in Fig 4 was made in a region where the ray had divided into four As can be seen in the figure the cut failed to sever all four subrays, the pair above and the upper member of the lower pair being the only ones fully cut The branches of the nerves in these subrays undoubtedly suffered a corresponding fate and consequently the axis of the denervated dark band does not correspond exactly to that of the group of four subrays but centers more toward what in Fig 4 is the upper pair This is clearly seen in the state of the melanophores distal to the cut (to the right in Fig 4) where above the upper pair of subrays they are fully dispersed and below the lower pair fully concentrated This is more *obvious in Fig 5, a photograph taken somewhat more distal to the* cut than Fig 4 and showing fully dispersed melanophores above the upper pair and fully concentrated ones below the lower pair

These are the conditions of the melanophores peripheral to the cut What is their state central to it? This can be seen in the left half of Fig 4 and in all of Fig 3 which includes the left part of Fig 4 and still more in the basal direction of the ray A comparison of the upper and the lower parts of these two figures shows indubitably that the melanophores above the upper pair of subrays have their pigment more dispersed than those below the lower pair do The lower melanophores are indeed almost punctate, the upper ones almost stellate The melanophores then central to a cut have slightly more dispersed pigment than those of the adjoining regions though the dispersion is by no means so pronounced as that of the denervated melanophores peripheral to the cut This is clearly seen by a comparison of the upper part of Fig 3 with the upper part of Fig 5 The condition in Fig 3 is naturally explained by assuming antidromic impulses from

the cut   It is not clear, however, why the nerve impulses are not as effective centrally as peripherally   This question, however, can be elucidated by further experimentation

It has already been pointed out that in *Fundulus* the melanophore nerves include two sets of opposing fibers, dispersing and concentrating   These fibers differ physiologically one from the other in that those concerned with dispersion are much more easily stimulated by being cut than those concerned with concentration, in other words, the dispersing fibers have a much lower threshold for stimulation by cutting than the concentrating ones do   Hence in a pale *Fundulus* these two sets of fibers are in very different states on the peripheral and the central sides of a denervating cut   Peripheral to the cut the dispersing fibers are highly excited and the concentrating ones are cut off from their normal centers of excitation   Central to the cut the dispersing fibers also highly excited antidromically must, however, oppose the concentrating fibers which in a pale fish are at a high degree of efficiency from their natural centers   Consequently the action of the dispersing fibers is felt by the melanophores less on the central side of a cut than on its peripheral side and this contrast is so marked in a pale *Fundulus* that scarcely any dark band at all is perceptible on the central side of the cut   The correctness of this view can be demonstrated by the following experiment

In carrying out a test to this end it is necessary to exclude from the central side of a given cut the activity of the concentrating nerve fibers and yet to maintain a pale condition in the fish as a whole   This can be attained by a double operation   If a denervating cut is made near the root of the tail of a pale *Fundulus* a peripheral dark band will develop as usual and if the fish is now kept in a white, illuminated tank for a few days, this band will fade almost to extinction   The conditions of the melanophores in such a faded band are shown in Fig 7 taken just peripheral to the cut and in Fig 8 from a position half way between that of the cut and the edge of the tail   The melanophores in these two regions are in an approximately stellate or stellate-punctate condition and in this respect agree essentially with those immediately central to the cut as shown in Fig 6   The band having attained this condition of semi-blanching is now cut for a second time and in a region midway between the first cut and the edge

of the tail (11–12 in Fig 2) Such a new cut in the position described will, as is well known, revive the peripheral part of the old band and since the central part of this band, at least as far as to the first cut, is now protected by that cut from the action of the concentrating fibers, this part of the band may be expected also to be revived provided the new cut calls forth antidromic impulses The results of the reviving cut thus favorably placed for the purpose of determining the presence of antidromic activities are shown in Figs 9 to 12 whose positions are indicated in Fig 2 (9 to 12) As is apparent in Fig 9 the melanophores central to the first cut have remained much as they were before the new cut was made (compare Fig 9 with Fig 6) Those peripheral to the new cut and shown in Fig 12 have again dispersed their pigment as was to have been expected But this renewed dispersion not only affects the melanophores of the band peripheral to the new cut, it also affects those central to it both next the new cut (Fig 11) and throughout the part of the band that lies between this cut and the first one (Fig 10) This region, between the two cuts, is the critical region It is now protected from the action of the concentrating fibers and if antidromic influences are present they should show themselves by a dispersion of pigment Such a dispersion is abundantly present Tests of this kind were made not only on the fish from whose tail the photographs shown in Figs 9 to 12 were taken, but on four other fishes all of which yielded similar results I, therefore, conclude that in the action of dispersing melanophore nerve fibers antidromic activities can be clearly demonstrated particularly if in advance these fibers are freed from the opposing action of the associated concentrating nerve fibers

To ascertain whether the concentrating melanophore nerve fibers show antidromic responses an entirely different procedure from that used with the dispersing fibers must be employed The concentrating fibers have a very low threshold for faradic stimulation as contrasted with the dispersing fibers Moreover there is no reason to suppose that after electric stimulation concentrating fibers remain active for any protracted length of time as the dispersing fibers do after having been cut From this standpoint concentrating fibers are much like ordinary motor fibers whose responses to electric stimulation are extremely brief Consequently illustrative photographs of the effects

of concentrating fibers in melanophores cannot easily be made because
of the transitoriness of these effects   Such effects, however, can be
very clearly demonstrated to the observer's eye

In order to test concentrating fibers in this respect it is desirable to
use killifishes that are only slightly dark   If they are fully dark their
skins are so filled with the dispersing neurohumor that the concen-
trating humor from the concentrating nerves cannot compete with the
opposing humor   Hence it is desirable to have only enough dispersing
humor present to give through its action on the melanophores only a
slightly dark tone to the skin   When on a *Fundulus* of appropriate
tint platinum electrodes from an inductorium are placed across one
of its caudal rays at the position seen in the cross line between A and B
in Fig 2, the electrodes may remain there indefinitely without exciting
any change in the fish   If now a faradic current is started through
them within a few seconds a pale spot will begin to appear under the
electrodes and spread rapidly as a band peripherally and centrally
over the ray   A quick inspection of the band under the microscope
shows that the pigment of the melanophores in the band has become
concentrated till each melanophore is in the punctate state   The
directions taken by the resulting blanching would apparently indicate
that the concentrating nerve fibers not only transmit peripherally but
also centrally, that is, that they exhibit antidromic activities   An
objection, however, might be urged against this interpretation of
the response in that it might be claimed that the blanching was not
nervous but due to a spread of the electric current, for, as a stimulus,
this is known to induce concentration of pigment   To check this
possibility slightly pale fishes were prepared by making denervating
cuts in their caudal rays and after the resulting dark bands had largely
faded out they were tested electrically as in the preceding trial
When in such a preparation electric stimulation was applied on the
peripheral side of the cut, the blanched area that resulted began at
the cut and extended peripherally from that region (Fig 2, B) and was
not to be seen central to the cut   If on the other hand the electrodes
were applied centrally to the cut, the blanched area started at that
point and spread centrally but not peripherally (Fig 2, A)   As the
cut in the ray interrupts the nerves but not the electrical conductivity
of the tissue, the spread of the blanching in these tests is to be attrib-

uted to nervous action and not to electric overflow   I, therefore, conclude that in *Fundulus* the concentrating melanophore nerve fibers, as well as the dispersing ones, exhibit antidromic activities and in this respect agree with other motor and sensory nerve fibers of the vertebrate body

SUMMARY

1 In *Fundulus heteroclitus* the dispersing melanophore nerve fibers have a relatively high threshold for faradic stimulation and a low one for stimulation by cutting   When they are protected from the competing action of the concentrating fibers, they show through the responses of their melanophores well marked antidromic activities which can also be seen to a slight degree even where the concentrating fibers are active

2 The concentrating melanophore nerve fibers in this fish have a relatively low threshold for faradic stimulation and a high one for stimulation by cutting   They also exhibit clear antidromic responses as shown by their associated melanophores

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EXPLANATION OF PLATES

The diagram and all photographs are from the killifish, *Fundulus heteroclitus*
The photographs were taken by Dr F M Carpenter

PLATE 2

FIG 1 Cleared preparation of a tail of *Fundulus* showing two bands, the one in the lower part of the tail came from a single cut, the other in the upper part originally from a single cut near the root of the tail reactivated by a second cut made near the middle of the length of the band and some days later than the first one

Fig 2   Diagram of a tail of *Fundulus* showing the positions of denervating cuts and also the positions at which the photographs in Plates 3, 4, and 5 were taken The numbers on this diagram refer to the figures on the succeeding plates   The uppermost band indicates the blanched area, A-B, due to stimulation of the concentrating nerve fibers by electrodes applied at the position of the cut but before it was made   Area B resulted from the application of the stimulating current peripheral to the cut, and area A when it was applied central to the cut

PLATE 3

Conditions of melanophores in a denervated band in the tail of *Fundulus* immediately after the band had been formed   See Fig 2
Fig 3   Immediately central to the denervating cut
Fig 4   At the cut
Fig 5   Immediately peripheral to the cut

PLATE 4

Conditions of melanophores in a denervated band in the tail of *Fundulus* some days after the band had been formed and shortly after it had faded   See Fig 2
Fig 6   Immediately central to the denervating cut
Fig 7   Immediately peripheral to the cut
Fig 8   On the band and midway between the cut and the edge of the tail

PLATE 5

Conditions of melanophores in a denervated band in the tail of *Fundulus*   The band had been allowed to fade and was then revived by a new cut   See Fig 2
Fig 9   Central to the first cut
Fig 10   Peripheral to the first cut but after the second cut had been made
Fig 11   Central to the second cut
Fig 12   Peripheral to the second cut
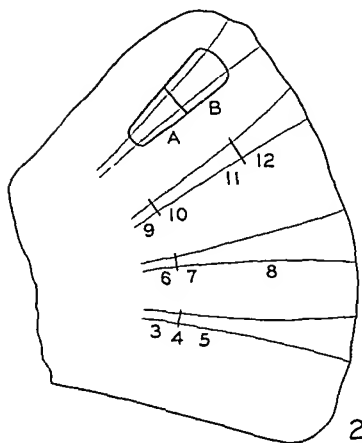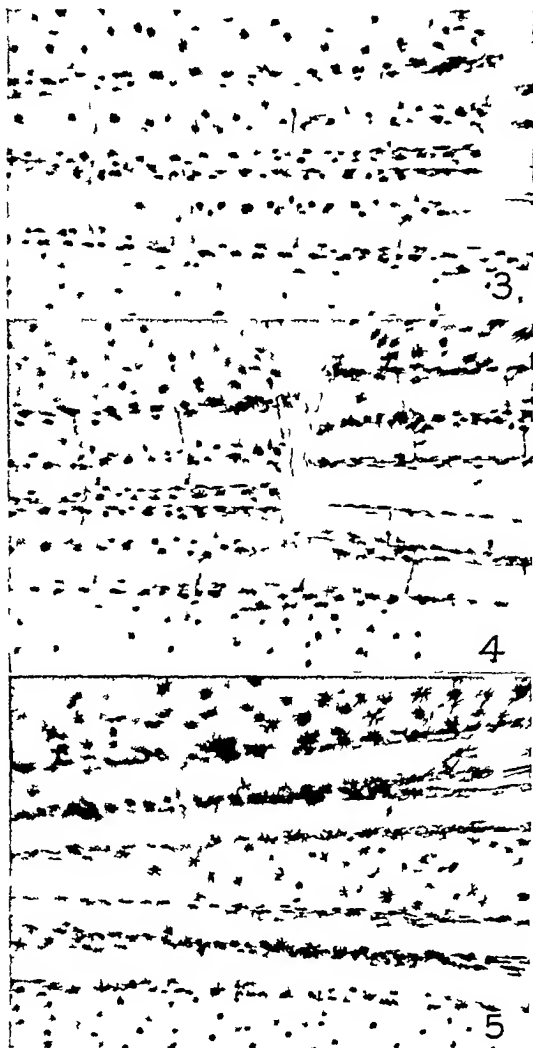
2

3.

4

5

(Parker Melanophore responses and a tidrmic responses)
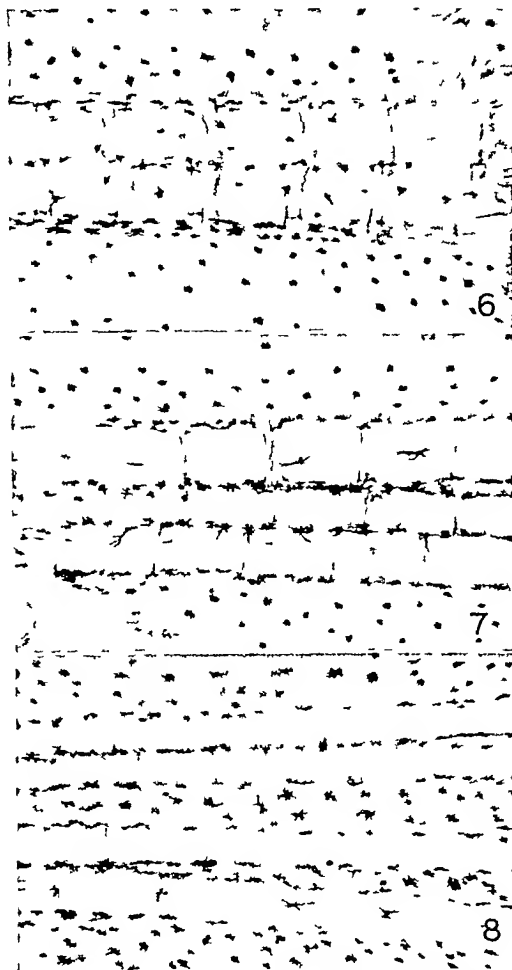
A PHASE RULE STUDY OF THE PROTEINS OF BLOOD SERUM THE EFFECT OF CHANGES IN CERTAIN VARIABLES*

By ELOISE JAMESON

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The quantitative measurements presented in the preceding communication (1) made with potassium citrate as a precipitant at pH 6 8 and 0°C on undiluted serum, differ greatly from the usual results found elsewhere in the literature The following experiments are now adduced to show that these differences arise from the circumstance that in most of the previous work not all of the essential variables, namely, the pH, temperature, kind of salt, and protein concentration, were controlled in the same manner

As a standard, curves made at pH 6 8 and 0°C with composite samples of blood serum from 30 or more male rats 100 days old were used, since different series were found to be reproducible and coincident Individual biological differences had thus been eliminated Any deviation from this standard could then be demonstrated In two cases a standard of females was substituted

In these solubility curves several fractions, A, B, C (C_1 and C_2), and D may be distinguished (1) The effect of change in each essential variable on these fractions has been determined

EXPERIMENTAL

Change in pH

The methods of procedure and analyses were the same as those described in a preceding paper unless otherwise mentioned

Previously, a pH of 6 8, as determined by the glass electrode, was used 5 5 was chosen for comparison with this because it is the pH used by Svedberg (2) as the isoelectric point of globulin

Serums from two different lots of 30 female rats were used   The
per cent of protein in the dialyzed serum, 6 62 per cent at a pH of 5 5,

TABLE I

Stock Diet

Blood Serum from Female Rats—5 Months Old, 0°C
pH 6 8

Total composition by weight		Liquid phase by weight	
Potassium citrate	Protein	Potassium citrate	Protein
per cent	*per cent*	*per cent*	*per cent*
19 86	5 770	20 00	5 666
23 82	5 473	24 50	4 527
25 78	5 326	26 95	3 937
26 79	5 252	27 70	3 556
27 78	5 177	28 96	3 218
29 75	5 030	31 07	2 608
31 72	4 883	33 23	1 876
32 70	4 808	34 45	1 592
33 78	4 727	35 95	0 935
34 66	4 660	36 87	0 869
35 63	4 585	37 70	0 511
37 55	4 437	39 75	0 133

pH 5 50

18 85	5 24	19 00	5 226
20 60	5 086	20 72	5 02
22 30	4 925	25 91	3 778
24 00	4 768	24 76	4 274
25 67	4 610	26 42	3 663
27 31	4 453	28 19	3 086
28 13	4 377	29 42	2 945
28 30	4 352	29 68	2 678
29 74	4 222	30 99	2 338
30 51	4 141	32 05	1 965
31 30	4 063	32 38	1 790
32 0S	3 987	31 97	1 972
32 84	3 908	34 11	1 301
33 59	3 830	34 90	1 054

was less than in the standard experiment, 6 95 per cent protein at
pH 6 9

The whole range of precipitation was not covered by the curve at

pH 5 5, since the curve at that pH did not take the shift to the left to be expected from the literature, a shift which would have shown a decrease in solubility of the protein fractions at the isoelectric point of globulin
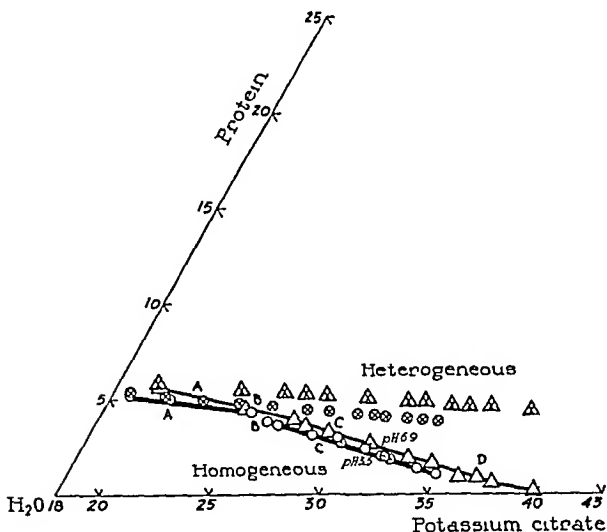
FIG 1 Comparison of curves at pH 5 5 and pH 6 9   Blood serum from 30 female rats   Phase rule diagram at 0 C

$\otimes$ = total composition pH 5 5
$\triangle$ = total composition pH 6 9
$\bigcirc$ = liquid phase pH 5 5
$\triangle$ = liquid phase pH 6 9

Table I and Fig 1 give the results of the precipitation of protein of rat serum at 0° by potassium citrate at the two different hydrogen ion concentrations, the standard curve at pH 6 8, the experimental curve at pH 5 5   As may be seen, in general the two curves follow the same slope except in the extreme left part   If lines are drawn connecting

the points of total composition in the two experiments and a ruler is placed across all four lines in their middle sections, the proportionality of the protein in solution to that of the total composition may be seen The conclusion may then be drawn that at 0°C the solubility of protein as denoted by the middle section of the curve is independent of the pH but dependent solely upon the total amount of protein present

There is less of the first fraction, A, and more of the second fraction, B, separating at a pH of 5 5   The break which is not apparent at a pH of 6 9 becomes visible at a pH of 5 5   This might be explained by the fact that part of the first fraction of protein is combined with lipoids by means of a bond which may be easily broken at the isoelectric point of the protein, changing its apparent solubility   A study of the solubility of this fraction in potassium citrate solutions is in progress

The qualitative effect of pH at room temperature is reported later in this article in the paragraph on salting out of proteins from diluted serum by $(NH_4)_2SO_4$ A distinct decrease in solubility from that shown in the standard curve occurs in the albumin end, at room temperature and probably even at 0°C

As may be seen from a comparison of the curves, the precipitation is not related to the citric acid in solution   In the experiments represented by points at pH 5 5 the citric acid was as much as 20 per cent of the amount of citrate when concentrations of citrate were highest In the curve for pH 6 8 the maximum is about 3 per cent

There are many observations in the literature upon the importance of pH control   In general it is known that the single proteins (3) are less soluble at their isoelectric points than at either higher or lower concentrations of H ions   In curves with whole serum very little change in solubility has been found between the pH's of 4 8 and 9 0(4) Butler, Blatt, and Southgate (5) have made a study of the effect of changing the pH on the complete salting out curve of serum with potassium phosphate   They find a parallelism between their curves at 39° with increasing solubility from pH 5 4 to 6 5 and to 7 7   However, they do not compare the curves at 0° where the effect of the salt on the proteins is minimal, but at 39° where the solubilities of the protein fractions have already been altered by the elevated tempera-

ture   Butler, Blatt, and Southgate have diluted the serum 1 to 16 or
1 to 31 with phosphate solution while in the experiments reported here
it is undiluted

The works of McFarlane (6) show clearly that existing bonds or
complexes are broken apart on dilution   Consequently, it is only in
undiluted serum that the proteins as they occur naturally, may be
studied

Change in Temperature

Two experiments were carried out as nearly simultaneously as
possible, on the same composite sample of female rat serum at the two
temperatures 0°C and 23°C   In each case the pH was 6 8

Table II gives the results, which are plotted in Fig 2

In general less protein remains in solution at 23° than at 0°C at a
definite salt concentration   The fraction A proves an exception to
this statement since the first portions of the two curves almost coin
cide   The solubility of A is, therefore, not as greatly affected by
temperature as by the change in pH just discussed

The quantity of fraction B becomes larger at 23° than at 0°   It is
not visible in female rat serum at 0°C   In the middle section of the
23° curve between the salt concentrations 24 5 per cent to 27 5 per
cent, approximately, there is almost no further precipitation of
protein with added increments of salt   For the first time, in this
work, the flat place so often described in the literature may be seen
The protein which precipitates gradually at 0° along the curve from
23 per cent to 27 5 per cent potassium citrate has come down suddenly
at 23° from 23 per cent to 24 5 per cent salt   It has become less
soluble, possibly due to aggregation

From 27 5 per cent of salt to the most concentrated solutions, there
is a gradual increase in the distance between the curves, indicating a
growing rate of precipitation at 23° until the protein is practically out
of solution at 36 per cent salt   The difference in solubility of proteins
with temperature is most marked in the albumin fraction, D   The
rise in temperature also increases the amount of fraction D as meas
ured by the difference between an extension of C and the base line

The effect of raising the temperature is, then, to cause a splitting
apart in the complex denoted by C with the simultaneous increase in
both fractions B and D

Many investigations have been carried out at low temperatures (7) as only by so doing may proteins be preserved unchanged for even a short time   The increase of solubility of proteins with decreased

TABLE II

Comparison of Influence of Temperature on Precipitation of Blood Serum from 60 Female Rats—5 Months Old—pH 6 8

Total composition by weight		Liquid phase by weight	
Potassium citrate	Protein	Potassium citrate	Protein
0°C			
per cent	*per cent*	*per cent*	*per cent*
19 86	5 770	20 00	5 666
23 82	5 473	24 50	4 527
25 78	5 326	26 95	3 937
26 79	5 252	27 70	3 556
27 78	5 177	28 96	3 218
29 75	5 030	31 07	2 608
31 72	4 883	33 23	1 876
32 70	4 808	34 45	1 592
33 78	4 727	35 95	0 935
34 66	4 660	36 87	0 869
35 63	4 585	37 70	0 511
37 55	4 437	39 75	0 133
Room temperature			
17 86	5 916	18 60	5 953
19 86	5 770	20 66	5 444
21 84	5 620	23 11	4 796
23 82	5 471	24 60	3 892
24 82	5 399	25 73	3 727
25 80	5 325	27 40	3 465
27 78	5 178	29 41	2 819
29 75	5 030	32 32	1 765
30 74	4 956	33 95	1 250
31 72	4 882	34 10	1 006
33 68	4 734	35 84	0 226
35 63	4 585	38 65	0 0143

temperature is also a matter of record (8)   The 23° curve shows a similarity to those of Butler and Montgomery (9) with phosphate solutions, and Howe's (10) with Na_2SO_4, for it might be drawn as a

senes of curved lines   In Butler and Montgomery's work, room temperature and in Howe's work a temperature of 37°C was chosen

Butler, Blatt, and Southgate's curves (5) show an increase in solubility of every fraction from 39° or 20° to 0° and a parallelism between
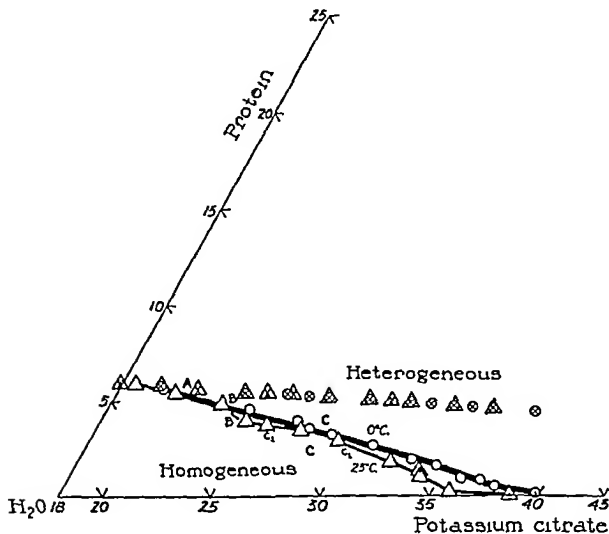
FIG 2 Comparison of curves at 0 C and 23°C   Blood serum from 60 female rats. Phase rule diagram

⊗ = total composition 0°C
○ = liquid phase 0°C
△ = total composition 23 C
△ = liquid phase 23°C

the curves   In their experiments complexes had already been split apart by dilution   It was then impossible to show the effect of a rise in temperature in accomplishing this alteration in the original state of the serum proteins

Changes in Nature of the Salt

Precipitation of male rat serum was carried out at 0° with $(NH_4)_2SO_4$ instead of potassium citrate as the precipitant without regard to keeping the pH constant   The pH changed from pH 6 9 in 15 per cent $(NH_4)_2SO_4$ solution to 6 3 in 29 per cent $(NH_4)_2SO_4$ solution (1) The results of the experiment are shown in Table III and graphed in Fig 3

The curve is of the same type as that made at pH 5 5 with potassium citrate in which the fraction A is lessened and fraction B is increased   The same straight line relationship of protein concentration to salt solution exists as with potassium citrate

TABLE III

Blood Serum from 30 Male Rats—100 Days Old—Precipitation by $(NH_4)_2SO_4$— 0°C —pH from 6 88 to 6 25

Total composition by weight		Liquid phase by weight	
$(NH_4)_2SO_4$	Protein	$(NH_4)_2SO_4$	Protein
per cent	*per cent*	*per cent*	*per cent*
14 86	5 847	15 11	5 510
15 80	5 782	15 66	5 388
17 70	5 652	17 92	4 550
19 60	5 521	19 93	3 840
21 50	5 391	22 14	3 020
23 41	5 260	24 40	2 105
25 32	5 128	26 77	1 072
27 24	4 997	29 02	0 260
29 16	4 865	31 09	Trace

Several different salts have been mentioned in the literature as suitable for salting out proteins   Sørensen (8) used $(NH_4)_2SO_4$ and acetates, Butler and Montgomery (9) used phosphates, Howe (10), Na_2SO_4 Hardy (7) found as early as 1910 that $(NH_4)_2SO_4$ is not suitable as a precipitant for euglobulin since the latter does not retain its original properties after being precipitated by that salt   In previous work, potassium citrate appeared to the author to be most satisfactory as a precipitant (1)

Change in Concentration of Protein

Table IV gives the data for the precipitation by potassium citrate of the proteins of rat serum at two different concentrations   The

data are plotted in Figs 4 and 5 The part of Fig 4 which shows the fusion of the curves is enlarged in Fig 5

Curve I, which is a composite made from two different lots of male rat serum under the standard conditions, is compared with curve II made from the same rat serum diluted 1 part serum to 2 parts 5 per
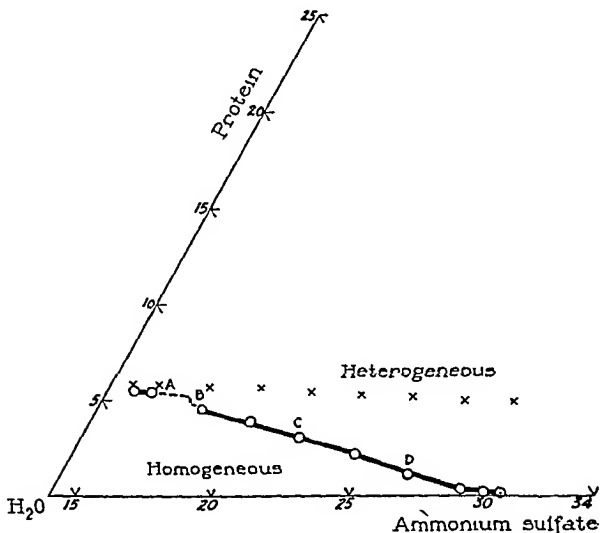
Fig 3 Diet—stock Blood serum from forty 100 day old male rats Phase rule diagram at 0°C

x = total composition
O = liquid phase

cent potassium citrate solution Except for the break which is not visible in curve II (possibly because its concentration is not great enough) the curves represent solubilities of proteins which are in proportion to the total compositions on the same tie lines until the change in direction occurs in curve I at 32 5 per cent salt From that point to between 37 5 per cent and 38 per cent salt, where the two

curves meet, curve I approaches curve II   At the latter point very little protein except the albumin fraction D is still in solution   Within the limits of error of the experiment the protein in solution is constant from 38 per cent salt to 41 per cent salt whether a concentrated or diluted serum is used for precipitation   Possibly enough solid phases are present to determine the protein content of the liquid phase   It is

TABLE IV

Comparison of Undiluted and Diluted Blood Serum from 40 Male Rats—100 Days Old Precipitated by Potassium Citrate 0°C —pH 6 8

Total composition by weight		Liquid phase by weight	
Potassium citrate	Protein	Potassium citrate	Protein
Undiluted Experiment 1			
per cent	*per cent*	*per cent*	*per cent*
19 61	5 412	19 85	5 314
23 58	5 135	24 03	4 529
25 57	4 997	25 84	3 883
26 57	4 925	27 22	3 605
27 56	4 857	27 84	3 616
29 54	4 718	30 17	2 881
31 51	4 580	32 19	2 371
32 49	4 510	33 69	1 765
34 46	4 372	36 02	1 088
35 43	4 301	37 10	0 734
37 36	4 160	39 54	0 1472
Experiment 2			
20 63	5 339	20 30	5 056
21 58	5 271	21 78	4 778
22 62	5 221	22 30	4 745
23 49	5 140	23 55	4 623
24 45	5 071	24 53	4 135
25 41	5 006	23 02	4 601
27 30	4 872	27 58	3 502
29 24	4 737	29 83	3 062
31 16	4 602	32 13	2 467
33 07	4 468	34 15	1 646
34 97	4 330	36 37	0 883
36 86	4 191	38 99	0 205
37 81	4 122	39 78	0 096
38 74	4 052	40 56	0 026

TABLE IV—*Concluded*

Total composition by weight		Liquid phase by weight	
Potassium citrate	Protein	Potassium citrate	Protein
Diluted 1 part serum to 2 parts potassium citrate solution Experiment 1			
per cent	*per cent*	*per ce 1*	*per cent*
21 82	1 712	21 95	1 595
23 81	1 667	23 85	1 392
25 83	1 616	26 08	1 348
26 78	1 600	26 97	1 241
27 77	1 577	27 86	1 139
29 74	1 533	30 29	0 940
31 68	1 487	32 31	0 786
32 68	1 464	33 66	0 727
33 66	1,432	34 32	0 627
34 68	1 419	35 11	0 593
35 61	1 396	36 18	0 496
37 53	1 351	38 47	0 268
38 49	1 328	39 17	0 1797
Experiment 2			
37 07	1 358	37 76	0 352
38 02	1 336	38 88	0 221
38 98	1 313	39 97	0 114
39 92	1 290	40 73	0 065

also possible, however, that the greater solubility of the fraction, D, may mask the small differences in solubility of some other protein fractions which are not single substances   The albumin, D, must exist as an independent protein of definite solubility, although its solubility is probably influenced by the presence of other proteins

Many studies on the solubility of the precipitated fractions of serum or plasma as individual proteins in respect to the effect of protein concentration have been carried out   Sørensen (8) showed a definite solubility in salt solution independent of the original protein concentration in the case of crystalline egg albumin, but found a fractiona tion when the solubility of globulin was investigated (11)   Fibrinogen and hemoglobin have definite solubilities, also, as proved by the excellent work of Florkin (12) and Green (13)

It should be noted that curve II made from diluted serum is not

comparable to those of Butler and his coworkers for the dilution has not been great enough to break the continuity of the curve C
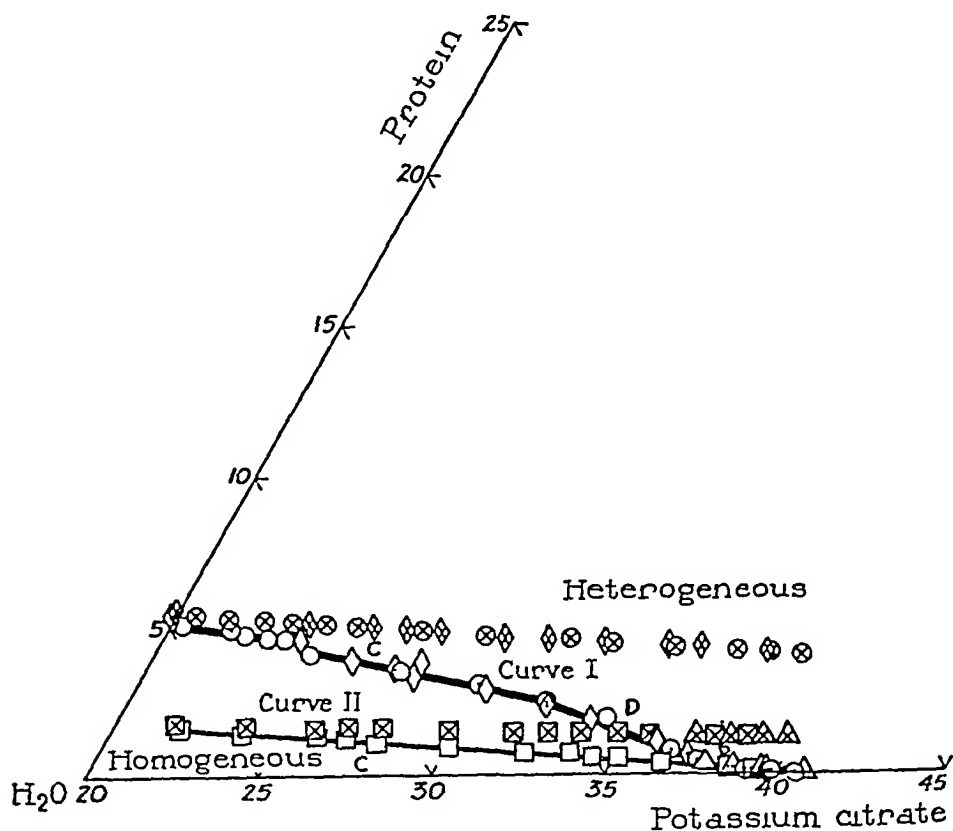
FIG 4 Diet—stock   Effect of dilution   Blood serum from 40 male rats
Phase rule diagram at 0°C
   Total composition

$\diamondsuit$ = undiluted samples
$\otimes$ = undiluted samples
$\triangle$ = diluted samples
$\boxtimes$ = diluted samples

   Liquid phase

$\diamondsuit$ = undiluted samples
$\bigcirc$ = undiluted samples
$\triangle$ = diluted samples
$\square$ = diluted samples

Effect of Change in Protein Concentration and Nature of the Salt

Proteins of male rat serum, diluted 1 to 10 with 5 per cent $(NH_4)_2SO_4$ solution were precipitated with $(NH_4)_2SO_4$ at 0°C   The pH of samples

changed from 7 1 in the more dilute to 7 0 in the concentrated solution
Table V and Fig 6 present the data   B is not visible, C has moved to
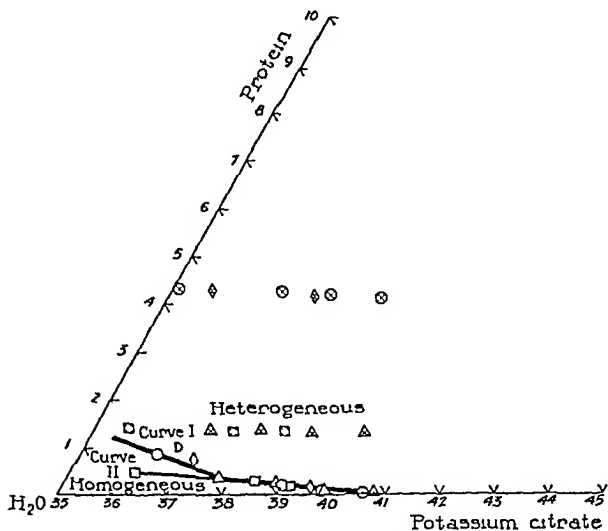
FIG 5 Diet—stock.   Effect of dilution   Blood serum from 40 male rats
Phase rule diagram at 0°C
Total composition

⬦	= undiluted samples
⊗	= undiluted samples
△	= diluted samples
⊠	= diluted samples

Liquid phase

◇	= undiluted samples
○	= undiluted samples
△	= diluted samples
□	= diluted samples

the left, and a flat place comes into view between C and D   At this
dilution no overlapping of C and D is discernible   An albumin

TABLE V

*Blood Serum from 100 Male Rats—100 Days Old   Diluted 1 to 10—Comparison of
Precipitation by (NH₄)₂SO₄ at 0°C and at Room Temperature*

Total composition by weight		Liquid phase by weight	
(NH₄)₂SO₄	Protein	(NH₄)₂SO₄	Protein
0°C —Average pH 7 10			
per cent	per cent	per cent	per cent
15 71	0 6001	15 65	0 6155
16 70	0 5930	16 61	0 5788
17 70	0 5859	17 59	0 5424
21 70	0 5575	21 57	0 3830
23 69	0 5432	23 78	0 3025
25 69	0 5290	25 60	0 2235
27 69	0 5148	27 59	0 1572
28 69	0 5077	28 80	0 1330
29 69	0 5006	29 90	0 0701
30 69	0 4935	30 51	0 0415
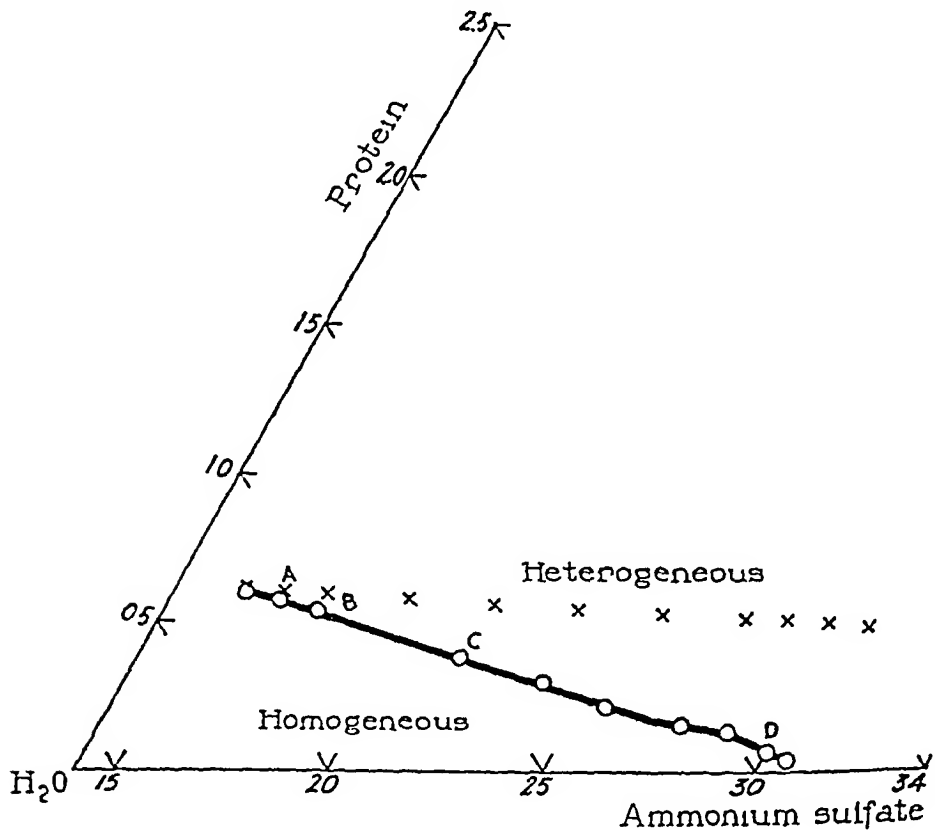
Fig 6 Diet—stock   Serum diluted 1–10   Blood serum from 30 male rats
Phase rule diagram at 0°C

x = total composition
O = liquid phase

TABLE VI

Blood Serum from 100 Male Rats—100 Days Old Diluted 1 to 10—Comparison of Precipitation by $(NH_4)_2SO_4$ at 0°C and at Room Temperature— 23°C—Average pH 7 01

Total composition by weight		Liquid phase by weight	
$(NH_4)_2SO_4$	Protein	$(NH_4)_2SO_4$	Protein
per cent	*per cent*	*per cent*	*per cent*
14 71	0 6071	14 53	0 607
15 71	0 6001	15 50	0 606
17 70	0 5859	17 69	0 550
19 70	0 5718	19 58	0 440
21 70	0 5575	21 57	0 369
23 69	0 5432	23 75	0 296
25 82	0 5281	25 85	0 199
27 69	0 5148	27 71	0 129
28 69	0 5077	28 68	0 0741
29 69	0 5006	29 62	0 04902
30 69	0 4935	30 72	0 01960
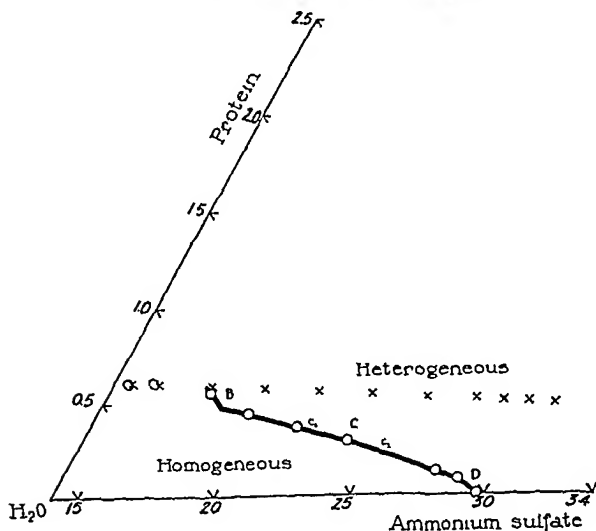
FIG 7 Diet—stock. Serum diluted 1–10 Blood serum from 30 male rats Phase rule diagram at 23°C

x = total composition
O = liquid phase.

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globulin ratio made by means of half saturation with $(NH_4)_2SO_4$ at this dilution would manifest a larger globulin and consequently a smaller ratio than one made with undiluted serum, a fact noted by Butler and coworkers

In determining the protein, the clear samples of the liquid phase were brought colorimetrically to a pH of 5 5 at room temperature In the tubes, more concentrated with respect to salt, a heavy precipitation of protein took place   It was almost completely resoluble at 0°C indicating, qualitatively, that at room temperature a change of pH affects the solubility of the albumin fraction conspicuously

Effect of Change in Protein Concentration, Nature of Salt and Temperature

With $(NH_4)_2SO_4$ as the precipitant of serum, diluted 1 to 10 with 5 per cent $(NH_4)_2SO_4$ solution, at room temperature the expected curve materialized   The curve in Fig 7 is plotted from the data in Table VI   A is largely missing, B is increased, probably at the expense of both A and C   A break in C becomes visible   There is present, then, both the effect of the raised temperature on C and the curve to the right of it and the effect of the $(NH_4)_2SO_4$ on A   Again, this curve could be drawn as a series of curved lines

DISCUSSION

The effect of changes in the variables on each protein fraction will be discussed separately

Fraction A (Probably Euglobulin) —Hardy and Gardiner (7) and Chick and Martin (3), found that the first fraction separating on adding salt was a combination of a protein and lipoid   Hardy observed that the solubility of this protein fraction in saturated NaCl solution could be decreased in two ways, namely by extraction with ether and by previously salting it out with $(NH_4)_2SO_4$ In the experiments described above its solubility curve has been reduced from that in potassium citrate solution at 0° and pH 6 8, by the use of $(NH_4)_2SO_4$ and by changing the pH to 5 5   It is possible that the solubility of fraction A has been changed by splitting off some of its combined

lipoid [1] Neither the change in temperature from 0° to 23°, nor dilution had any appreciable effect on A

Fraction B —The amount of fraction B, as indicated in the standard curves, may be augmented either when the solubility of A is changed to the isoelectric point of globulin, pH 5 5, or when $(NH_4)_2SO_4$ is used, or when the fraction, C, is decreased at room temperature These additions to fraction B may or may not be identical

Fraction C —When the temperature of precipitation is changed from 0° to room temperature, not only is the production of B and possibly D at the expense of C apparent but there is a break in the ordinarily straight line denoting the separation of phase C Since fraction C has been broken up into two or more constituents or components it is probably a complex substance This hypothesis is in accord with all of our results The curve in Fig 6 gives an explanation for McFarlane's (6) observation that the globulin fraction increases on dilution of serum The two components of C have separated from each other in part That these components may recombine is suggested by his experiments in which there is an increase in the number of molecules of the size of albumin on mixing albumin and globulin solutions The globulin particles may be aggregated when uncombined with albumin and redispersed when albumin is added Hardy's (14) observation that globulin present in serum will pass through a porous pot which holds it after precipitation and resolution in alkali or salt solution, is further confirmation of this conception

Fraction D —The last fraction to separate on salting out, D, is definitely a single protein in high concentrations of salt, as proved by its constant solubility regardless of the amount of protein in the original mixtures Its solubility is definitely less at room temperature than at 0°

In this work we have been concerned with the number of the protein fractions and whether they are individuals or not We have determined that each of at least four different fractions, A, B, C, and D may be altered independently of the others This fact is an indication

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[1] The combination may be disturbed in some cases of glomerular nephritis where an alteration in the cholesterol content of the serum occurs At least the fraction A is largely missing although the total globulin is not reduced

of the individuality of the fractions although not a proof that one or more of them may not be a combination of two or more proteins or even continuous series of compounds

The charges of the proteins as well as the salt concentrations enter into the solubilities of the individual fractions as Cohn's formula indicates   The effect of charge on solubility is negligible in those cases in which the pH can be changed without altering the solubility

CONCLUSIONS

Changes were studied in the standard solubility curve of fresh serum proteins by alterations in pH, temperature, concentration of protein, and nature of the salt used for precipitation

The principal factor affecting the precipitation of protein fractions was a change in temperature   In order to investigate the proteins in their original states low temperatures are necessary

Protein fraction A is altered by a change in pH and with the use of $(NH_4)_2SO_4$ as a precipitant, fraction B by a change in pH and temperature, and use of $(NH_4)_2SO_4$, C by a change in temperature and concentration of the protein, and D by a change in temperature and pH

The solubility of D is independent of the amount of protein in solution in high concentrations of salt

Throughout the duration of this border line study of the application of the phase rule to a physiological problem, I have been indebted to Dr T Addis and Dr J W McBain for helpful criticism

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THE BIOGENESIS OF PRIMARY SEX HORMONES

I THE FATE OF ESTRINS INJECTED INTO THE RABBIT*

BY GREGORY PINCUS AND PAUL A ZAHL

(From the Biological Laboratories, Harvard University, Cambridge)

The rabbit normally excretes scarcely detectable quantities of estrogenic material (Pincus, Wheeler, Young, and Zahl (1936)), but in jected estrin may be recovered in the urine (Smith and Smith (1931)) Smith and Smith have in fact shown that 600–700 r u (rat units) of theelin injected into non pregnant rabbits results in the excretion of about 30 r u over a period of 4 days after injection, whereas the simultaneous injection of 600–700 r u of theelin plus progestin into an ovariectomized rabbit doe results in the recovery of about 500 r u of estrogenic material in the urine   This difference may be due to the transformation of theelin (estrone) into a more active estrogen in the progestin treated animal, or to a greater destruction of the estrone in the non pregnant animal with no change in its chemical constitution

Estrone might conceivably be converted into four native estrogenic compounds in the organism, estradiol, estriol, equilin (and its isomer hippulin), and equilenin (Fieser (1936))   Of these, by ordinary test, estradiol is more active than estrone, estriol and equilenin less active, equilin and hippulin about as active   Since estradiol is the ovarian hormone and estrone a urine component it seems likely that the latter is an excretion product and not normally converted to the former, furthermore, estradiol has been recovered from the urine of mares in small amount only (Wintersteiner, Schwenk, and Whitman (1935)) while estrone is obtained in large amount   The equilinic compounds have been obtained from members of the horse family only, so that it seems unlikely that rabbits would produce them   Nonetheless since the nature of rodent estrogens is unknown we cannot ignore the possibility

Fortunately there exists a specific color test for estriol which should

make possible its detection in urine extracts   This is the production of a blue color when the hormone is heated with concentrated H_2SO_4 followed by cooling, dilution, and further heating with arsenic acid (David (1934))   In a previous paper (Pincus, Wheeler, Young, and Zahl (1936)) we demonstrated that when human urines are separated into estrone and estriol fractions after the method of Cohen and Marrian (1934) the David test cannot be employed for estriol determinations because of the presence of inactive interfering substances Furthermore, due to the inactive materials in rabbit urines all ordinary colorimetric tests give a positive value for both estrone and estriol when neither are in fact present   By modifying the extraction procedure we are now able to free rabbit urines from the interfering chromogens so that the David test can be employed as a quantitative measure of estriol and other tests as a measure of the hormone content of the estrone fraction

Methods

Urine collections have been made from rabbits (1) in heat, (2) pseudopregnant, (3) pregnant, (4) hysterectomized in heat, (5) hysterectomized pseudopregnant, (6) ovariectomized   48 hour samples are taken since we have found that these give comparable volumes   Two 48 hour urine samples are taken preceding the injection of estrogenic material and urine is collected after injection as long as hormone is excreted   The urine is extracted and fractionated according to the following procedures

1   Acidified to pH 2 0 with HCl and placed in an autoclave at 15 lbs pressure for 15 to 30 minutes

2   Extracted three times with ethyl ether

3   Ether evaporated and residue dissolved in toluol   Ether extract washed three times with 20 per cent Na_2CO_3 solution   Washings discarded

A   B

Washed three times with N/10 NaOH to remove the "estriol" fraction
Hydroxide solution acidified with CO_2
Extracted three times with ethyl ether
Washed with 20 per cent Na_2CO_3 solution until interface between Na_2CO_3 solution and ether is perfectly clean
Ether evaporated and residue taken up in 95 per cent ethanol (estriol)

Washed three times with 1 N NaOH to remove the "estrone" fraction
Hydroxide solution acidified with CO_2
Extracted three times with ethyl ether
Washed with 20 per cent Na_2CO_3 solution until interface between Na_2CO_3 solution and ether is perfectly clean
Ether evaporated and residue taken up in 95 per cent ethanol (estrone)

This is essentially the procedure of Cohen and Marrian (1934) except that the hydrolysis under pressure is carried on for a very short interval of time   Washing is made twice with 20 per cent Na_2CO_3 rather than once with 10 per cent Na_2CO_3 and fractionation is made from a toluol extract (A) rather than the ethereal extract they use   Acid hydrolysis for the 2 hours they employ almost completely destroys the hormone in rabbit urines   The washing with 20 per cent Na_2CO_3 removes interfering chromogens and pigmented materials, so that our final solutions are colorless (Schmulovitz and Wylie (1935))   The fractionation is made from toluol because this reagent retains certain pigmented chromogens that are not retained by ethyl ether

For rapid determinations of hormone content we employ the red color obtained by diazotization with sulfanilic acid   The reagents are (1) 5 gm sulfanilic acid dissolved in 50 cc. concentrated HCl diluted to 500 cc with distilled H_2O, (2) 25 gm $NaNO_2$ in 500 cc distilled H_2O (3) 15 per cent NaOH in aqueous solution The color reagent is made up by adding 1 5 cc of the $NaNO_2$ solution to 5 cc of the sulfanilic acid solution and diluting to 50 cc with H_2O This reagent gives a constant color reaction with the hormones at between 1/4 and 2 hours after it is made up   Fresh reagent must be made after 2 hour intervals or longer   The color reaction is obtained by adding exactly 2 cc of the color reagent to 2 cc of alcoholic hormone extract   After thorough mixing 3 drops of 15 per cent NaOH are added The red color resulting remains constant for 1/2 hour and then grows somewhat more intense, so that readings of color absorption are made within 1/2 hour using the S 51 filter of the Pulfrich photometer   The hormone content is determined from a standard curve made with crystalline hormone   We make up a new standard curve for each set of determinations since the color reaction varies from day to day   These standard curves do not differ by more than 25 per cent, but this is too wide a variation to justify the use of a single standard

The colorimetric assay of the estriol fraction is checked by the David test (Table II)   We do not employ this test for rapid determination since it is much more time-consuming   It should be used however, to make certain of the estriol content since the sulfanilic acid test will reveal the presence of phenolic compounds other than the hormone [1]   There is no specific test for estrone comparable to the David test, but the sulfanilic acid determinations have been checked by the modified Cohen and Marrian phenolsulfonic acid test described by Pincus, Wheeler, Young and Zahl (1936)   This test is about five times as sensitive as the sulfanilic acid test and will reveal small quantities of material not detectable by the sulfanilic acid test   On the other hand it will give positive tests for inactive materials not revealed by the sulfanilic acid test

Equilenin is determined by the color reaction described by Sandulesco, Tschung and Girard (1933)

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[1] It should be noted that inactive chromogens are rarely encountered in our extracts   Their presence is always denoted by slight pigmentation of the extracts Removal of the pigment results in correct colorimetric titers

TABLE

The Estrogen Content of Various Rabbit Urines before and after the Injection of Estrone, and Estrone Benzoate, Using the ...

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
			Estrone content							Estriol content		
Rabbit No	Condition of rabbit	Amount of hormone injected	Days 4–3 before injection	Days 2–1 before injection	Days 1–2 after injection	Days 3–4 after injection	Days 5–6 after injection	Days ...	Days 3 before injection	Days 2–1 before injection	Days 1–2 after injection	Days 3–4 after injection
			micrograms	micrograms	micrograms	micrograms	micrograms	micrograms	micrograms	micrograms	micrograms	micrograms
1	Estrus	300 micrograms estrone	0	0	20	20	0		0	0	37	0
2	Pseudopregnant (1st 10 days of pseudopregnancy)	" "	0	0	38	20	0		0	0	100*	40*
3	Pregnant (1st 10 days of pregnancy)	" "	0	0	55	20	0		0	0	45	35
4	Pregnant (last 10 days of pregnancy)	" "	0	0	35	20	0		0	0	25	(0)
5	Hysterectomized (unmated)	" "	0	0	35	0	0		0	0	0	0
6	Hysterectomized (mated 1st 10 days of pseudopregnancy)	" "	0	0	105	38	20		0	0	0	0
7	Ovariectomized (1st 10 days after ovariectomy)	" "	0	0	35	10	0	-	0	0	33*	0
8	Ovariectomized (2 mos after ovariectomy)	" "	—	—	55	0	0	-	—	—	0†	0†
9	Estrus	300 micrograms estriol	0	0	0	0	0		0	0	33	0
10	Pseudopregnant (1st 10 days of pseudopregnancy)	" "	0	0	0	0	0		0	0	110*	42
11	Hysterectomized (unmated)	" "	0	0	0	0	0	-	0	0	33	15
12	Hysterectomized (mated 1st 10 days of pseudopregnancy)	" "	0	0	0	0	0	-	0	0	125*	50
13	Ovariectomized (1 mo after ovariectomy)	" "	0	0	0	0	0	-	0	0	45	15
14	Pseudopregnant (1st 12 days of pseudopregnancy)	600 micrograms estrone benzoate	0	0	130	51	37		0	0	126*	62
15	Hysterectomized (mated)	" "	0	0	168	125	75		0	0		
16	Ovariectomized (1½ mos after ovariectomy)	" "	0	0	110	100	75		0	0	0† / 0	0 / 0

* Positive David test
† Negative David test

ol, and Estrone Benzoate, Using the Sulfanilic Acid Test for Urinary Hormone

(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)
Estriol content						Total hormone excreted		Total estrin (estrone plus estriol)	Injected hormone recovered
Days 4-3 before injection	Days 2 1 before injection	Days 1 2 after injection	Days 3-4 after injection	Days 5-6 after injection	Days 7 8 after injection	Estrone	Estriol		
micrograms	micrograms	micrograms	micrograms	microg ams	micrograms	micrograms	micrograms	micrograms	per cent
0	0	37	0	0	—	40	37	77	25 7
0	0	100	40*	0	—	58	140	198	66 0
0	0	45	35	0	—	75	80	155	51 7
0	0	25	60	0	—	55	85	140	46 7
0	0	0	0	0	—	35	0	35	11 7
0	0	0	0	0	—	163	0	163	54 3
0	0	33	0	0	—	45	33	78	26 0
—	—	0†	0†	0†	—	55	0	55	18 3
0	0	33	0	0	—	0	33	33	11 0
0	0	110	42	0	—	0	152	152	50 7
0	0	33	15	0	—	0	48	48	16 0
0	0	125*	50	8(?)	—	0	183	183	64 3
0	0	45	15	0	—	0	60	60	20 0
0	0	126*	62	0	0	218	188	406	93 8
0	0	0†	0	0	0	423	0	423	97 9
0	0	0	0	0	0	340	0	340	78 7

RESULTS

The data on urinary estrin as determined by the sulfanilic acid test are presented in Table I   It is immediately deducible that estrone as such or in the form of the benzoate is converted to estriol in the rabbit and that estriol is not converted but excreted unchanged   But the conversion of estrone does not occur in hysterectomized rabbits (Nos 5, 6, 15), nor in rabbits long ovariectomized (Nos 8, 16) Shortly after ovariectomy a limited amount of conversion occurs (No 7) This implies that the conversion takes place in the uterus, but that it will not take place in a uterus lacking ovarian control   Since in no instance have detectable amounts of hormone been obtained before injection (Table II) it seems safe to conclude that the excreted material is derived from the injected material unless one postulates that the injected estrogens stimulate the endogenous production of excretion products   Such stimulation is scarcely credible in view of the excretion by ovariectomized animals since the ovary is presumably the hormone precursor, secondly, one would have to assume further that estriol stimulates only estriol excretion and estrone the excretion of both in certain animals and not in others, a most unlikely situation

The data demonstrate also that a two- to fourfold increase in excretion of injected estrone and estriol excretion products occurs in animals with corpora lutea in the ovaries (Nos 2, 3, 4, 6, 12)   This may occur if the presence of a corpus luteum (1) prevents destruction or (2) hastens excretion

The injections of estrone monobenzoate were undertaken to test this point   That the benzoylation of the estrone prevents its destruction in the liver has been demonstrated by Zondek (1934) working with mice   The estrone benzoate-injected animals received 600 micrograms of the compound of which 433 micrograms were estrone The data demonstrate again that the conversion to estriol occurs only in an animal with a functional uterus   The mean percentage of hormone recovered for the three animals of this group is 90 1 with the lowest value (78 7 per cent) for the ovariectomized animal (No 16) This lowest value in the ovariectomized female would indicate that the corpus luteum may prevent destruction, since about 21 per cent of the protected molecules in this case disappeared in this animal compared with 6 per cent in the pseudopregnant female (No 14), and 2 per cent in the hysterectomized pseudopregnant animal (No 15)

It will be noted, however, that the complete excretion of the estrone took place within 6 days in the pseudopregnant animal (No 14) and in 8 days in the other two (Nos 15 and 16) This would indicate more rapid excretion by the pseudopregnant animal It seems possible, therefore, that the presence of a corpus luteum increases the excretion of injected hormone and thus prevents its destruction This conclusion is not especially supported by the data on the "free" hormone injections, since excretion is ordinarily completed in 4 days in animals without as well as with corpora lutea, the sole difference seeming to reside in the absolute quantities of hormone excreted Thus in the estriol injected animals the mean quantity of hormone excreted during the second 48 hour period after injection is 10 micro grams per animal for the three animals without corpora lutea, the ratio of excreted hormone in the two groups is 1 4 6, whereas for the preceding 48 hour period it is 1 3 2 The corresponding quantities in the estrone injected series are 7 5 and 58 micrograms for the second 48 hours and 53 and 98 micrograms for the first 48 hours with ratios of 1 7 7 and 1 1 8 respectively, indicating in both estrone and estriol recipients relatively more hormone excretion in the second 48 hour period, e g , therefore less destruction

In Table II we present the data on a number of these extracts, comparing the hormone content as determined by various colori metric tests and by bioassay Since the David test is specific for estriol the presence of other materials giving a positive sulfanilic acid test will be revealed by the sulfanilic acid test, giving a higher titer than the David test In five extracts (Nos 1, 3, 10, 12, 18) the sulfanilic acid test gives a higher titer, in three (Nos 2, 2, 11) the David test titer is higher On the average the sulfanilic acid test gives a slightly higher titer This may be due to (1) inactive chromo gen, (2) small amounts of estrone in the estriol fraction, (3) small amounts of estradiol The bioassays should reveal the presence of the latter since by our tests it is many times as active as estriol The three estriol fractions (Nos 2, 14, B_2) titered by bioassay indicate more hormone than either the sulfanilic acid or David tests reveal If we deduce that there is estradiol present in these extracts the amounts of estradiol would be 6, 14, and 2 micrograms respectively

In the case of the estrone fractions the sulfanilic acid titers and phenolsulfonic acid titers agree fairly well, except that the

TABLE II

The Hormone Content of Various Rabbit Urine Extracts As Determined by Several Colorimetric Tests and by Bioassay

Rabbit No	Extract* No	By sulfanilic acid test	By phenolsulfonic acid test	By bioassay	By David test	
		micrograms	*micrograms*	*micrograms*		
1	5	0	0	—		
1	6	20	31	—		
2	4	0	0	—		
5	5	0	0	—		
6	4	0	0	—		
6	6	105	164	154		
7	4	0	0	—		Estrone
8	6	55	42	—		
10	4	0	0	—		
13	4	0	0	—		
14	6	130	—	150		
15	4	0	0	—		
16	9	55	50	—		
B.†	—	0	—	0		
1	11	0	0	—	0	
1	12	37	—	—	25	
2	10	0	0	—	—	
2	12	100	—	167	120	
2	13	40	—	—	45	
3	10	0	0	—	—	
3	12	45	—	—	30	
5	11	0	0	—	—	
6	10	0	0	—	—	
7	10	0	0	—	—	Estriol
10	10	0	0	—	—	
10	12	110	—	—	105	
11	12	33	—	—	40	
12	12	125	—	—	105	
13	10	0	0	—	—	
14	12	126	—	233	—	
15	10	0	0	—	—	
B₁†	—	—	—	0	0	
B.†	—	—	—	120	102	

* The extract No refers to the column number of Table I, *e g* rabbit No 1, extract No 6, refers to the estrone determination made on rabbit No 1 extract taken 1–2 days after estrone injection

† These extracts are not included in the data of Table I, B₁ is the unheated extract from a rabbit receiving 300 micrograms of estrone, B₂ is from the same batch of urine heated according to our regular technique

to give slightly higher values   This is to be expected since the phe
nolsulfonic acid test reveals small amounts of inactive materials that
act as chromogens   Comparison of colorimetric values with bioassay
values reveals an excess of activity in one case (No 14) and approxi-
mate equivalence in two others (Nos 6 and B_2)   If the excess is due
to estradiol, No 14 contains less than 4 micrograms of estradiol

We have tested a number of these extracts for equilenin by the
Girard test   The result is invariably negative   Therefore the rabbit
produces no equilenin   Since equilenin in urine is invariably accom
panied by equilin or hippulin (Fieser (1936)) we may deduce that
these compounds are not formed

The conversion of estrone to estriol that these experiments demon-
strate occurs only in a functional uterus   Two possibilities seem
evident (1) the ovary produces an hitherto unidentified hormone
which acts upon the uterus to effect the conversion, or (2) the corpus
luteum hormone stimulates the uterus in such a way as to make this
conversion possible   It can scarcely be estrogenic hormone since we
would expect a larger conversion than observed from the estrus female
(No 1) and conversion in the ovariectomized animal No 8 which
received estrogen   The second alternative mentioned above is on the
face of it rendered unlikely by the conversion observed in the estrus
rabbit (No 1), and in the recently ovariectomized rabbit (No 7),
unless we postulate that the estrus rabbit's ovary produces corpus
luteum hormone in the absence of formed corpora lutea and that in
the recently ovariectomized animal the effect of this luteal hormone
on the uterus lasted for some days after ovariectomy

To test these alternatives we performed the experiments the data
of which are given in Table III   The five rabbits of these experi
ments were given a preliminary injection of 300 micrograms of estrone
The first 48 hour collection was made from the end of the 2nd to the
end of the 4th day after this injection in order to determine the hor-
mone excretion occurring just before a second injection of 300 micro
grams of estrone given simultaneously with 0 5 mg of progesterone
in oil   The first injection of estrone served also to prime the uterus
of the ovariectomized females   In one ovariectomized rabbit (No
21) the second injection contained no progesterone, so that it might

TABLE III

The Effect of the Injection of Progesterone upon the Excretion of Urinary Estrin in Estrone-Injected Animals

300 micrograms of estrone were injected 48 hours before the collection of urine was begun, and 48 hours after the first collection an additional 300 micrograms of estrone were injected simultaneously with 0 5 mg of progesterone

Condition of rabbit	Estrone content in urine specimen				Estriol content in urine specimen				Total hormone excreted after injection		Total estrogen (estrone + estriol)	Increase of excretion over animal not receiving progestin
	1	2	3	4	1	2	3	4	Estrone	Estriol		
	micrograms	micrograms	micrograms	micrograms	micrograms	micrograms	micrograms	micrograms	micrograms	micrograms	micrograms	per cent
17 Estrus	20	35	25	0	0	85	0	0	70	85	135	75 3
18 Pseudo pregnant	15	38	20	0	65	90	0	0	58	90	148	25 3*
19 Hysterectomized (unmated)	0	33	25	0	0	0	0	0	58	0	58	65 7
20 Ovariectomized (4 mos)	25	20	15	12	0	45	0	0	47	45	92	95 7
21 Ovariectomized† (2 mos)	15	47	0	0	0	0	0	0	47	0	47	14 5*

* Per cent decreased excretion
† No progesterone in second injection

serve as a control for animal No 20 Urine specimens Nos 2, 3, and
4 in Table III represent urines wherein the effect of progesterone is
evidenced The estriol data of Table III were checked by the David
test so that there can be no doubt that animal No 20 did convert
estrone to estriol whereas its control did not This supports the
notion that the conversion takes place in a uterus influenced by
progesterone The data on the hysterectomized rabbit (No 19) in
which no conversion occurred indicate again that the uterus is
necessary

In addition we see from these data that progesterone facilitates
the excretion of additional estrogen In the last column of Table III
the increased percentage excretion is based upon the data of Table I
Thus animal No 17 (Table III) excreted a total of 135 micrograms of
estrin whereas animal No 1 (Table I) excreted 77 micrograms, indi-
cating an increase of 75 3 per cent in the former over the latter The
increase in the hysterectomized rabbit (No 19) was 65 7 per cent, in
the ovarectomized rabbit (No 20) 95 7 per cent (over No 21) The
pseudopregnant female (No 18) showed small decrease when com
parison is made with the control rabbit (No 5) but this is compre
hensible when we consider that the initial estrone injection presumably
inhibited or suppressed the effect of the animal's own corpus luteum
secretion The decrease in animal No 21 (compared with No 8)
is within the limit of error of these determinations

DISCUSSION

The data of these experiments support the scheme of estrogenic
hormone conversion *in vivo* that follows

I
Estradiol

II
Estrone
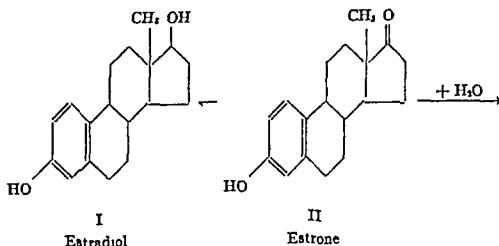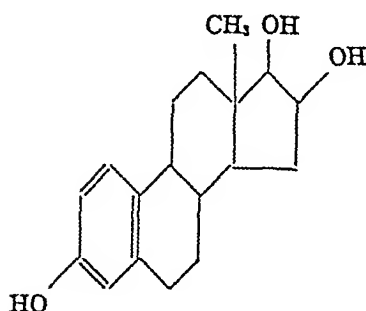
III
Estriol

The step from II to III is an irreversible hydration, and is the chief result of estrone injection   The step from II to I occurs presumably only in limited amount, if at all, or if this is an extensive conversion only small amounts of the diol are excreted   The former statement would seem most probable since the animals receiving estrone benzoate and excreting most of the injected hormone excrete chiefly estrone and estriol (see Nos 14, 15, 16 especially, in Table II)

The conversion II to III has not been accomplished in the laboratory, although Butenandt and Hildebrandt (1931) have obtained estrone by the dehydration of estriol   The conversion II to I has been made by Schwenk and Hildebrandt (1933) and Girard, Sandulesco, and Fridenson (1933) by catalytic reduction   According to Mac-Corquodale, Thayer, and Doisy (1936) estradiol is the ovarian hormone and we would therefore expect I to II to occur more easily than the reverse since estrone is an excretion product, estradiol not

These data leave no doubt that the conversion II to III requires a functional uterus, and that the action of progesterone on the uterus facilitates the reaction   That corpus luteum secretion normally promotes the conversion is somewhat indicated by the fact that estrone-injected animals with corpora lutea and uteri (Nos 2, 3, 4) excreted more estriol (305 micrograms total) than estrone (188 micrograms total), whereas those in that group without corpora lutea (Nos 1 and 7) excreted more estrone (85 micrograms total) than estriol (70 micrograms total)   Animal No 14, however, reverses the expected excretion on this basis   This may be due to the possibility that estrone benzoate has a low kidney threshold compared to the

kidney threshold of the bound form of estrone excreted by estrone injected animals or to a lesser reactivity of the molecule in the benzoate compound

It should be clearly understood that the actual nature of the compound involved in the steps II to III or II to I is not known, e g, whether "free" or "bound" hormone is the reactant Since we must employ a mild hydrolysis to obtain full recovery from the urine we know that bound hormone is excreted and to judge by analogy with human urines probably as a glycuronic acid compound (Cohen and Marrian (1936)) But the binding appears to be different than in the compounds in human urine since the rabbit urines require a much less drastic hydrolysis

These experiments support the notion that corpus luteum hormone causes increased estrin excretion by preventing destruction of the hormones rather than by lowering the kidney threshold, and lend credence to the opinion of Smith and Smith (1936) that "there is no renal threshold for estrin " We are thus led to the apparently paradoxical situation in which one of two competing hormones protects its competitor At the same time it should be noted that progesterone facilitates the conversion of estrone into the less active estriol During early pregnancy, for example, when a high concentration of estrin might very well lead to abortion the sterilizing potency of estriol is about one fourth to one fifth that of estrone (Pincus and Kirsch (1936)) Furthermore, we do not know just what chemical form these hormones take in vivo, and it may well be possible that the protection afforded by progesterone action involves the production of an estrin compound which is physiologically inactive Since the liver is the presumable site of estrin destruction, and the liver enzyme system presumably acts chiefly upon the phenolic portion of the estrin molecule (i e, benzoylation prevents destruction) progesterone might act to induce something like benzoylation of the phenolic ring Alternatively (or perhaps at the same time) progesterone might replace estrin in the liver system due to a higher partition coefficient of the progesterone for the particular system involved That progesterone may be more quickly destroyed than the estrins is indicated by the data of Table III which show no excretion of estriol in urine specimens 3 and 4 whereas the estrone continues to be excreted 48 to 96 hours after estriol excretion is completed If the estriol excretion be taken

as an index of the presence of active progesterone (particularly in animals Nos 17 and 20), it would seem that the 0 5 microgram progesterone injected was more rapidly destroyed than the 0 3 microgram of estrone injected at the same time

SUMMARY

1   A method is given for the extraction and fractionation of rabbit urines which frees these urines of inactive chromogens but permits a quantitative recovery of estrone and estriol for the colorimetric determination of these compounds

2   Estrone and estriol content of rabbit urine extracts can be determined by the concentration of the colored compound they form upon diazotization with sulfanilic acid and by the modified phenolsulfonic acid test of Cohen and Marrian   Estriol can be determined by the specific reaction first described by David   The technique for these tests is presented

3   Estriol (300 micrograms) injected into rabbits (*a*) in heat, (*b*) pregnant, (*c*) pseudopregnant, (*d*) hysterectomized in heat, (*c*) hysterectomized pseudopregnant, (*f*) ovariectomized, is excreted in the urine as estriol   Rabbit does in the luteal phase (*b*, *c*, and *c*) excrete 3 to 4 times the amount of estriol excreted by females without corpora lutea (*a*, *d*, and *f*)

4   When estrone (300 micrograms) is injected into the same types of rabbit does types *a*, *b*, and *c* excrete both estrone and estriol, type *f* excretes both estrone and estriol shortly after ovariectomy, but only estrone at 2 months after castration   Hysterectomized animals (types *d* and *c*) never excrete estriol after estrone injection   The total urinary estrin (estrone plus estriol) in estrone-injected animals is increased 2 to 3 times in animals in the luteal phase (*b*, *c*, and *c*)

5   It is concluded that the uterus is the site of conversion of estrone to estriol, and that the conversion cannot take place in a uterus completely free of ovarian control (*e g*, in long time ovariectomized animals)

6   In neither estrone-injected nor estriol-injected females is all the injected hormone recovered in the urine   The maximum recovery is 66 per cent   When estrone-benzoate (600 micrograms) is injected 94–98 per cent of the hormone is recovered from animals in the luteal phase (types *c* and *c*) and about 79 per cent in an ovariectomized

female (type f)   These data are taken to indicate that luteal secretions give partial protection against destruction to the hormones

7 The observation that in certain of the urine extracts the hormone titer by bioassay is somewhat higher than the colorimetric titer may indicate that there is a slight conversion of estrone to estradiol, particularly since no equilenin was found in any of the extracts by colorimetric test

8 The simultaneous injection of 300 micrograms of estrone and 500 micrograms of progesterone 4 days after an initial injection of 300 micrograms of estrone results in (1) an increased estrin excretion in females in heat, hysterectomized unmated, and ovariectomized, and a slight decrease in the pseudopregnant female, (2) the appearance of estriol in the urine of the long time ovariectomized animal with no urinary estriol in a control ovariectomized animal receiving no progesterone   These findings are taken to prove that the conversion of estrone to estriol occurs in the uterus under the influence of progesterone   Since animals in heat produce small amounts of estriol after estrone injection it is inferred that the ovaries of estrus rabbits produce small amounts of corpus luteum hormone in the absence of formed corpora lutea

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